THE ROLE OF BACTERIAL PROTEASES IN THE DEVELOPMENT OF CHRONIC WOUNDS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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Abbreviations

ANOVA.....Analysis of variants

AS.....Ammonium sulfate

BCM.....Biofilm-conditioned medium

bFGF.....Basic fibroblast growth factor

CXCL12.....C-X-C motif chemokine 12

DGGE.....Denaturing gradient gel electrophoresis

DFP.....Diisopropyl fluorophosphates

ECM.....Extracellular matrix

EDTA.....Ethylenediaminetetraacetic

EGF.....Epidermal growth factor

EGT.....Exuberant granulation tissue

ELISA.....Enzyme-linked immunosorbent assay

EPCs.....Endothelial progenitor cells

EPS.....Extracellular polysaccharide substances

ETT.....Endotracheal tube

FGF.....Fibroblast growth factor

FISH.....Fluorescence *in situ* hybridisation

H&E.....Haematoxylin and eosin

HCV.....Hepatitis C virus

HIV.....Human immunodeficiency virus

HNE.....Human neutrophil elastase

IFN-γ.....Interferon-gamma

IGF-1.....Insulin-like growth factor-1

IL-1β.....Interleukin-1 beta

MHA.....Mueller Hinton Agar

MHB.....Mueller Hinton broth

MMP.....Matrix metalloprotease

MRSA.....Methicillin-resistant *Staphylococcus aureus*

MT.....Masson's trichrome

MT-MMP.....Membrane type-matrix metalloproteinase

NEM.....N-ethylmaleimide

NPT.....Negative pressure therapy

PBMCs.....Peripheral blood mononuclear cells

PCM.....Planktonic-conditioned medium

PDGF.....Platelet-derived growth factor

PDT.....Photodynamic therapy

PMN.....Polymorphonuclear leukocytes

PMSF.....Phenylmethylsulfonyl fluoride

PNA-FISH.....Peptide nucleic acid-based fluorescence *in situ* hybridisation

RT-PCR.....Reverse transcriptase polymerase chain reaction

SDF-1.....Stromal cell-derived factor 1

SEM.....Scanning electron microscopy

TGF-β.....Transforming growth factor-beta

TIMP.....Tissue inhibitors of metalloprotease

TLCK.....1-chloro-3-tosylamido-7-amino-2-heptanone HCl

TLR.....Toll-like receptor

TNF-α.....Tumour necrosis factor-alpha

VAP.....Ventilator-associated pneumonia

VEGF.....Vascular endothelial growth factor

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Abstract

A large number of bacteria are able to secrete extracellular proteases that play vital roles in nutrient acquisition and biofilm formation but are also important biochemical mediators in bacterial virulence, facilitating host invasion. Microorganisms within chronic wounds have been hypothesised to form biofilms, which result in perpetuated inflammation and delayed wound closure. Whilst it is thought that exaggerated secretion of host-derived proteases within chronic wounds prevents successful wound closure, the contribution of bacterial proteases secreted from planktonic microorganisms and biofilms in chronic wound pathology has yet to be elucidated.

It is therefore the primary research aim of this thesis is to assess the proteolytic activity of *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from equine and human chronic wounds and determine whether there is a difference in activity between planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM). The next aim will be to identify these bacterial-derived proteases using zymography and mass spectrometry, and determine whether these proteases reduce wound closure in *in vitro* scratch wound models. More specifically, the effect of *P. aeruginosa* and *S. aureus* PCM and BCM from equine and human clinical isolates, and also purified proteases of these preparations, on the *in vitro* wound closure of equine normal fibroblasts (NFs), equine chronic wound granulation tissue fibroblasts (GTFs) and human dermal fibroblasts (HDFs), shall be investigated. In these wound models, zymography will be utilised to determine the release of host-derived matrix metalloproteases (MMPs).

In this thesis I have identified, for the first time, high protease activity in equine chronic wound-derived *P. aeruginosa* PCM and BCM, by which 52kDa and 42kDa proteases were detected. *P. aeruginosa* PCM and BCM were shown to significantly reduce the wound closure of NFs ($P < 0.0001$) and GTFs ($P < 0.0001$). Furthermore, the soluble products of *P. aeruginosa* biofilms, but not planktonic *P. aeruginosa*, elicited the release of metalloproteases from equine NFs and GTFs. In human studies, high protease activity specific to *P. aeruginosa* BCM but not PCM ($P < 0.0001$) was

determined. *P. aeruginosa* BCM significantly reduced the wound closure of HDFs ($P < 0.0001$) and cell viability ($P < 0.0001$), and further induced the release of metalloproteases from HDFs. *P. aeruginosa* in biofilm form secreted 62kDa and 52kDa proteases, however, through the partial purification of these proteases, it was determined that these proteases did not play a role in the reduction of wound closure in HDFs. The *P. aeruginosa*-derived partially purified proteases did however reduce HDF cell viability ($P < 0.05$).

The results in this thesis support the theory that bacterial-derived proteases may contribute to the emphasised proteolytic environment of human chronic wounds. Furthermore, the presence of bacterial biofilms within chronic wounds may induce the release of host-derived proteases. However the role of these specific *P. aeruginosa*-derived bacterial proteases in chronic wound pathology remains undetermined.

Chapter 1: Introduction

1 Introduction

Cutaneous tissue repair is a complex biological process that has been well researched in terms of the cellular mechanisms that take us from initial tissue injury to granulation tissue formation, successful tissue remodelling and re-epithelialisation. Despite this, the mechanisms involved in the development of chronic, non-healing wounds have not been fully elucidated. A wound that fails to heal in a timely manner or results in compromised tissue integrity is defined as a chronic wound (Stadelmann et al. 1998). Common examples of chronic wounds in humans include pressure ulcers, venous ulcers and diabetic foot ulcers, which pose a considerable economic burden, costing the National Health Service (NHS) an estimated £2.3-£3.1 billion per year (Posnett and Franks 2008). The physiological processes of tissue repair in chronic, non-healing wounds are disturbed and are halted in the inflammatory phase, manifesting clinically as persistent inflammation, increased tissue degradation, and excessive exudate and malodour (Gardner et al. 2001). Whilst there are a number of physical factors that are thought to lead to the development of a chronic wound, including reduced blood flow in the elderly and diabetic, vasculitis, mechanical pressure and burns; evidence of localised imbalances in protease production is thought to be one theory as to why wounds fail to heal within these patient groups. Matrix metalloproteases (MMPs) play a fundamental role in all aspects of tissue repair. Essentially, the primary role of these proteases is to cleave components of the extracellular matrix (ECM), a process that is carefully regulated by tissue inhibitors of metalloproteases (TIMPs). MMPs also play alternative roles in the release of growth factors and activation of other proteases.

The recognition of microbial biofilms in the context of the healthcare-associated infections has come to the forefront in recent years due to its association with increased antimicrobial resistance (Donlan 2001). Biofilms can be described as complex communities of microorganisms that can attach to each other and both biotic and abiotic surfaces, and reside within extracellular polysaccharide substances (EPS) (Donlan 2002). Biofilms have been associated with various pathologies including cystic fibrosis, endocarditis and periodontal disease (Paju and Scannapieco 2007, Nallapareddy et al. 2006). Indeed, the presence of bacterial biofilms in chronic

wounds has also been recognised and it has been hypothesised that the presence of biofilms within the wound could be a contributing factor to delayed wound healing (James et al. 2008). Despite the amplified proteolytic milieu observed in chronic wounds and the presence of bacterial biofilms, the effect of bacterial proteases on wound closure and indeed their contribution in the pathogenicity of chronic wounds has not been researched.

The aim of this chapter was to summarise mammalian wound healing, drawing attention to the similarities and differences between chronic wounds in both humans and horses. Furthermore, the role of endogenous MMPs in physiological and pathophysiological tissue repair human shall be reviewed. I have paid special attention to the involvement of biofilms in delayed wound healing and the potential role of bacterial proteases in the development of chronic wounds.

1.1 Wound Healing

1.1.1 Physiological Cutaneous Tissue Repair

Cutaneous tissue repair in humans comprises of a series of overlapping events that occur in order to clear contaminating bacteria and cellular debris and to produce new, vascularised, granulation tissue and a protective epithelial barrier. The wound healing process can be categorised into three major phases: haemostasis and inflammation, re-epithelialisation and granulation tissue formation and finally, matrix remodelling (Epstein et al. 1999).

Upon initial tissue injury, the extravasion of blood components into the wound leads to haemostasis through the initiation of the coagulation cascade and constriction of blood vessel walls. The process of clot formation and the infiltration and aggregation of platelets helps to prevent blood loss. The subsequent de-granulation of platelets within the clot results in the release of alpha granules (α-granules), which secrete growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), and platelet factor-IV. The release of these growth factors not only initiates

the coagulation pathways leading to fibrin clot formation, but also initiates the inflammatory phase of tissue repair through the recruitment of several cellular mediators (Stadelmann et al. 1998).

Within the early phase of inflammation, within 24-36 hours of tissue injury, neutrophils are the first cells to infiltrate the wound site through a number of chemoattractants including cleaved ECM components, TGF-β, complement proteins and bacterial-derived formyl-methionyl peptide products (Velnar et al. 2009). The primary role of neutrophils in the wound site is to phagocytose invading bacteria and foreign material. However earlier research has shown that the presence of neutrophils at the wound site is not a necessary cellular component in wound repair (Simpson and Ross 1972). Within this time frame, epithelial cells at the wound edge begin to migrate and proliferate along the dermis, forming a basement membrane. The later stages of inflammation, within 48-72 hours, are characterised by the recruitment of monocytes in response to cytokines and growth factors including TGF-β, PDGF and platelet factor IV, but also cleaved fragments of collagen and elastin. The monocytes soon differentiate into tissue macrophages. These macrophages then dominate this phase of wound repair, phagocytosing invading bacteria and causing local debridement (Leibovich and Ross 1975). Interestingly, research by Martin and colleagues (2003) has since shown that the infiltration of inflammatory neutrophils and macrophages is not essential for wound healing. This study showed scarless wound healing in the PU.1 null mouse, a model lacking both neutrophils and macrophages, in which wound healing was comparable to wild-type siblings and cellular debris was alternatively engulfed by fibroblasts (Martin et al. 2003).

Following haemostasis and an appropriate host immune response, the wound enters the proliferative phase of tissue repair. The proliferative stage of wound healing, occurring between day 3 and week 2, is predominated by the fibroblast-driven deposition of ECM and the subsequent generation of granulation tissue (Velnar et al. 2009). Fibroblasts are recruited to the wound due to the presence of factors such as TGF-β and PDGF, whereby they then proliferate and produce matrix proteins including fibronectin, hyaluronan, collagens and proteoglycans. The production of new granulation tissue brings about the formation of new blood vessels, a process

known as angiogenesis, from pre-existing vessels at the wound site. At this stage of physiological wound repair, epithelialisation is complete.

The lengthiest phase of the wound repair process is tissue remodelling, also referred to as 'maturation', which can take up to several years. Within this phase, there is a vascular regression of many of the newly formed capillaries formed in the proliferative phase, and thus vascular density returns to normal. There is a constant synthesis and breakdown of ECM components, particularly collagen, until the tissue architecture of the wound resembles that of normal tissue (Velnar et al. 2009, Guo and DiPietro 2010).

1.1.2 Chronic Wounds: The pathological mechanisms

The implications of infection within chronic wounds are numerous, including delayed wound healing, increased patient discomfort, increased rates of morbidity and mortality and increased costs of treatment (Reynolds and Chow 2013). In chronic, non-healing wounds, the normal responses to tissue damage are impaired and, wound healing is thought to be halted in the inflammatory phase, therefore a persistent state of inflammation is observed (Enoch and Price 2004). The mechanisms behind why some wounds fail to heal or take excessively long periods of time to heal is still unclear, however in humans, factors such as smoking, infection, reduced mobility, diabetes mellitus and nutrient deficiency are thought to increase the risk of developing a chronic wound (Guo and DiPietro 2010).

A number of cellular mechanisms have been described in the pathophysiology of chronic wounds. Firstly, the cellular activity of chronic wounds differs greatly from that of acute wounds. A common feature of chronic wounds is the failure of epithelialisation across the wound area, which is thought to be due to altered keratinocyte migration. For instance, Usui and colleagues assessed the expression of keratin-67 (a marker of keratinocyte proliferation) and LM-3A32 (a precursor for the alpha-3 chain of laminin 5 and a marker present in migrating epithelium) and found that keratinocytes in the margins of chronic diabetic ulcers, showed increased expression of keratin-67 and decreased LM-3A32 when compared to normal, acute

wound keratinocytes (Usui et al. 2008). Further to this, the proliferative capacity of cultured fibroblasts isolated from human diabetic foot ulcers was greatly reduced when compared with cultured fibroblasts from normal, healthy skin. Fibroblasts within diabetic foot ulcers are much larger in size and are spaced further apart in the tissue when compared to normal skin fibroblasts (Loots et al. 1999).

Host immune cells such as $CD3^+$ and $CD4^+$ T-cells have also shown to be significantly reduced in chronic diabetic and venous ulcers when compared to an acute wound model. Furthermore, $CD20^+$ B-cells, $CD79a^+$ plasma cells and $CD68^+$ macrophages are found at higher levels in chronic diabetic and venous wounds than acute wounds (Loots et al. 1998). A characteristic influx of neutrophils has been observed in chronic pressure ulcers, which leads to the release of neutrophil-derived proteases such as elastase and MMPs, contributing to ECM degradation (Nwomeh et al. 1999). For instance, neutrophil-derived elastase is known to degrade growth factors including PDGF and TGF-β, both beneficial growth factors for the release of ECM components from resident fibroblasts (Eming et al. 2007). Indeed, imbalances in the secretion of extracellular matrix (ECM) degrading proteases and the counteracting release of their inhibitors within chronic wounds has been well published (Menke et al. 2007, Widgerow 2011) and will be discussed in more detail in the next section of this introductory chapter.

Finally, the presence of bacteria within a chronic wound is thought to be a potential reason as to why chronic wounds will not heal. Bjarnsholt and colleagues (2008) hypothesised that it was the bacterial burden of a wound that prevents a chronic wound from healing, in particular the Gram-negative bacteria *Pseudomonas aeruginosa*. More specifically, they hypothesised that this was due to the ability of this microorganism to form a biofilm within the wound, which effectively is able to resist the action of antimicrobials, evade host immune responses and release virulence factors (Bjarnsholt et al. 2008).

1.1.3 Wound repair in the horse: A good model?

Studies into wound healing and aberrant wound healing generally use laboratory-bred models such as rabbits or mice, however there are considered to be great differences in these models when compared to wound healing in humans (Greenhalgh 2005). For example, commonly used mouse models have been heavily criticised as wound healing predominantly relies on muscle contraction due to the presence of the panniculus carnosus muscle in the subcutaneous tissue, unlike in humans, which lack this muscle and therefore wound healing is primarily driven by epithelialisation. Wound repair in the horse however, is steadily being acknowledged as a more physiologically relevant model for wound healing research (Theoret and Wilmink 2013).

Physiological wound repair in the horse is similar to that of human wound healing, consisting of an inflammatory phase, proliferative phase and a maturation phase (Stashak and Theoret 2009). Wounds on the trunk of the horse heal more rapidly through contraction than wounds on the lower limb, which rely more heavily on epithelialisation, resulting in slow-to-heal wounds and therefore drawing similarities to human chronic wounds (Knottenbelt 1997). Furthermore, equine lower-limb wounds can develop fibroproliferative complications as seen in humans. For instance, the spontaneous over-production of granulation tissue (exuberant granulation tissue), which leads to a protrusion of tissue with high fibroblast densities, is similar to that of human keloids (Theoret et al. 2013) (see **Figure 1.1)**.

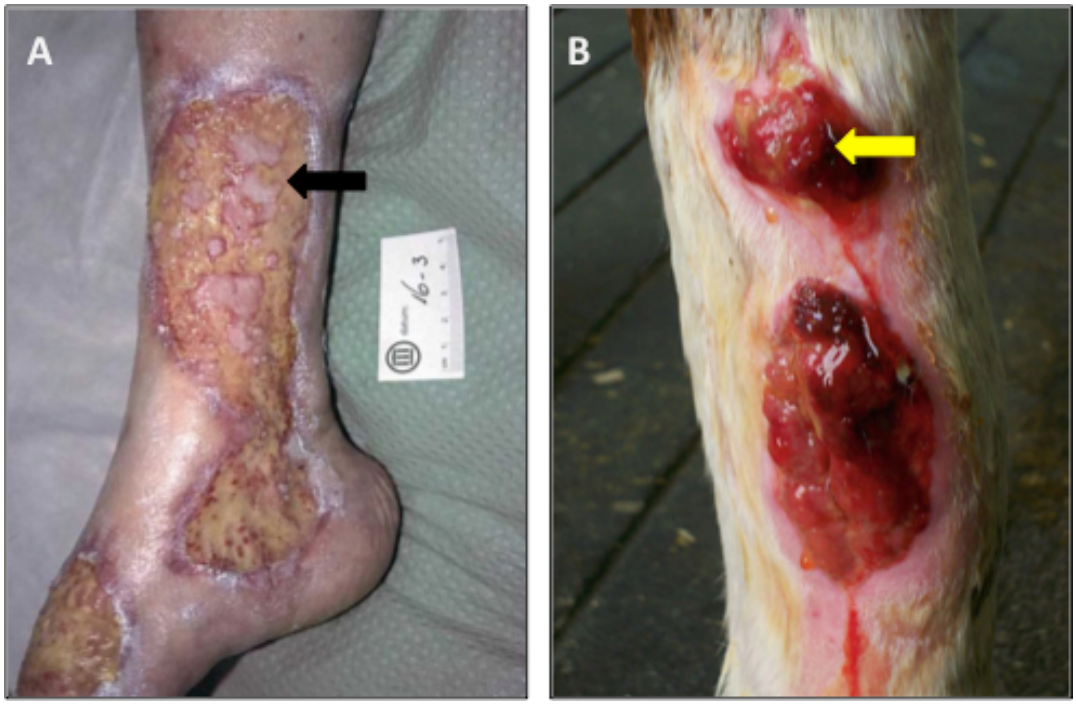


Figure 1.1 Images of human and equine chronic wounds.

Human chronic lower-leg wound (**A**) shows characteristic presence of slough, indicated by the black arrow, and the chronic equine lower-limb wound (**B**) shows exuberant granulation tissue, indicated by the yellow arrow. Permission to include these pictures was obtained from Dr Christine Cochrane (University of Liverpool).

1.2 Matrix Metalloproteases: An Introduction

Proteases are enzymes that are able to break down proteins and peptides and, depending on their activity, can be classified as either exoproteases or endoproteases. An exoprotease generally cleaves a peptide near the end of the N- or C-termini of the polypeptide chain and can be classified as amino- or carboxyproteases. An endoprotease, or proteinase, can cleave internal peptide bonds and are grouped into metallo-, serine-, aspartic- or threonine-proteases. To date, there are 569 known human proteases, the most abundant of which are metalloproteases, or matrixins, which account for 194 proteins, closely followed by

serine proteases which make up 176 of these proteins (Moali and Hulmes 2009). Matrix metalloproteases (MMPs) are a matrixin subfamily of zinc metalloproteases belonging to the M10 family of peptidases according to the MEROPS peptidase database (http://merops.sanger.ac.uk/cgi-bin/famsum?family=M10) (Rawlings et al. 2012). In humans, there are 23 known MMP genes (see **Table 1.1**) most of which are multi-domain proteins (Visse and Nagase 2003).

1.2.1 Molecular structure

MMPs belong to a group of peptidases known as 'metzincins', named after a conserved methionine C-terminal to the zinc ligands, known as a 'Metturn'. MMPs are grouped into gelatinases, collagenases, membrane-type (MT)-MMPs, matrilysins, stromelysins and others, based on their domain arrangement and substrate preference. MMPs all have a similar basic structure in that they all have a signal peptide, a pro-peptide and a catalytic domain. However they differ in the presence of fibronectin-type II repeats in the catalytic domain, as seen in the gelatinases MMP-2 and MMP-9 (see **Figure 1.2**). Furthermore, all MMPs except MMP-7 have a haemopexin domain that is involved in substrate recognition and some MMPs have an additional transmembrane domain with a cytoplasmic tail (MMP-14, -15, -16 and 24) or GPI anchor (MMP-17 and MMP-25) (Nagase et al. 2006). The pre-proenzyme contains a cysteine switch located in the pro-peptide and a zinc binding motif within the catalytic domain. The interaction between the conserved cysteine within the pro-peptide and the zinc ion in the catalytic domain keeps the pro-MMP inactive by preventing the binding of a water molecule (essential for catalysis) to the zinc atom (Nagase et al. 2006).

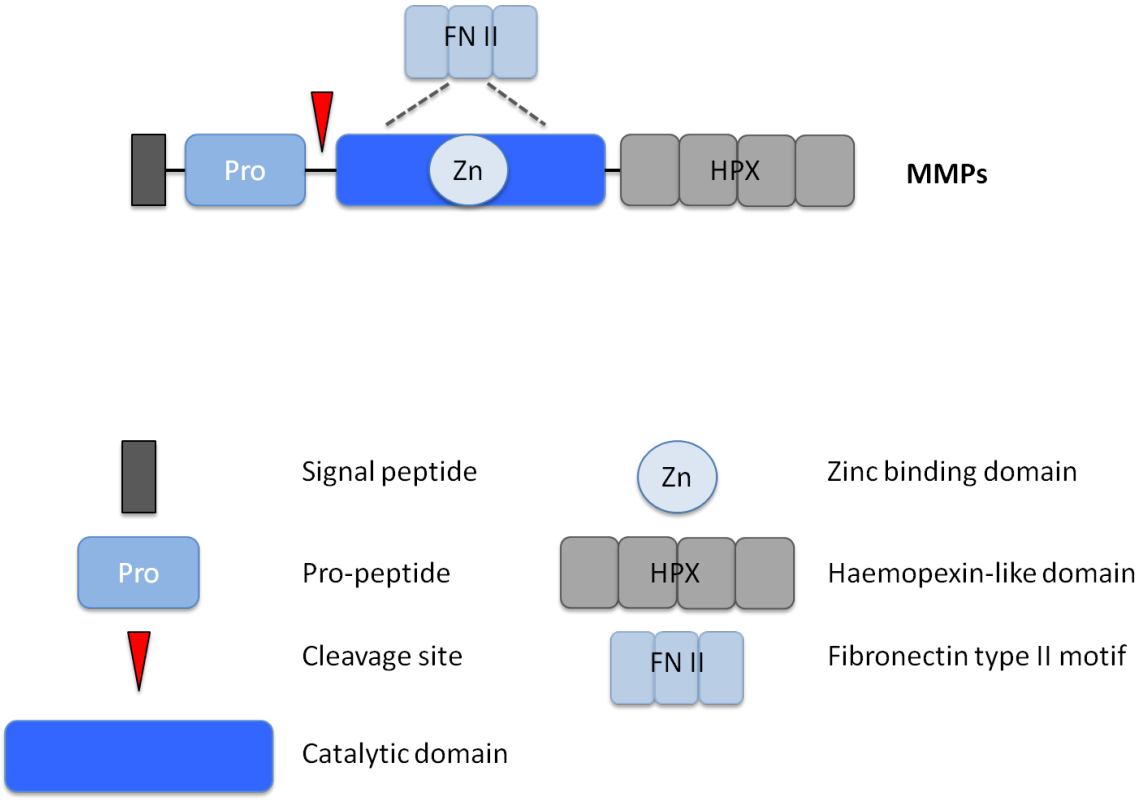


Figure 1.2 Schematic domain structure of human matrix metalloproteinases (MMPs)

MMPs generally consist of three domains, the pro-peptide, catalytic and a C-terminal haemapexin-like domain. The figure represents the inactive MMP precursor with a pro-peptide domain. MMP precursors are cleaved between the pro-peptide and catalytic domain, resulting in the release of the active enzyme.

1.2.2 Synthesis and activities

The synthesis of an MMP occurs within the cells and begins by the production of an inactive precursor, or an inactive pre-proenzyme. The signal peptide of the pro-MMP is cleaved intracellularly during translation. Many MMPs are secreted from the cell as pro-MMPs, whereby regions of the pro-peptide, known as 'bait regions', are susceptible to proteolytic cleavage by extracellular proteases. Often the cleavage of the pro-peptide by extracellular proteases is only partial, and therefore the complete

removal of the pro-peptide is carried out by other MMPs or through auto-catalytic cleavage, thus resulting in an active MMP. Some MMPs are activated intracellularly, whereby the inactive pro-MMP is proteolytically cleaved in the Golgi apparatus, resulting in an active peptide that can then be secreted. MMP activity can be regulated by Tissue Inhibitors of MMPs (TIMPs) -1, -2, -3 and -4, to variable extents. TIMPs are endogenous inhibitors belonging to the family I35 according to the MEROPS database (Rawlings et al. 2012), which can inhibit MMPs in a 1:1 stoichiometry, playing an important physiological role in tissue remodelling (Visse and Nagase 2003). TIMPs are able to bind to the zinc ion within the catalytic domain of active MMPs, thus expelling the water molecule bound to the zinc ion and inhibiting the MMP.

1.2.3 Biological function

The primary role of MMPs is to degrade components of the ECM including collagens, elastin and proteoglycans (see **Table 1.1**), mediating processes such as tissue remodelling and facilitating cell migration. However it has been recognised that the breakdown of ECM by MMPs also releases growth factors bound to the matrix, increasing growth factor availability to receptors. Furthermore, MMPs play a role in in other, equally as important biological processes through the cleavage of other substrates (Nagase et al. 2006). For instance, macrophage-derived MMP-9 has been shown to cleave the chemokine CXCL12 or stromal cell-derived factor 1 (SDF-1), which inhibits the recruitment of endothelial progenitor cells (EPCs), a key player in the promotion of vasculogenesis, in a wounded diabetic mouse model (Feng et al. 2014).

Enzyme name	MMP	Substrates
Interstitial collagenase/ Collagenase 1	MMP-1	Collagen I, II, III, VII, VIII, X, gelatin
Neutrophil collagenase/ Collagenase 2	MMP-8	Collagen I, II, III, VII, VIII, X, aggrecan, gelatin
Collagenase 3	MMP-13	Collagen I, II, III, IV, IX, X, XIV, gelatin
Gelatinase A	MMP-2	Gelatin, collagen I, II, III, IV, V, VII, X, aggrecan, elastin, fibronectin
Gelatinase B	MMP-9	Gelatin, collagen I, III, IV, V, elastin, aggrecan
Stromelysin 1	MMP-3	Collagen II, IV, IX, X, XI, gelatin, elastin, aggrecan,
Stromelysin 2	MMP-10	Collagen IV, elastin, aggrecan, laminin, fibronectin
Matrilysin 1	MMP-7	Fibronectin, laminin, collagen IV, gelatin, elastin
Matrilysin 2	MMP-26	Gelatin, casein, fibronectin, fibrinogen
Stromelysin 3	MMP-11	Collagen IV, fibronectin, laminin, aggrecan
MT1-MMP	MMP-14	Gelatin, fibronectin, laminin, aggrecan, casein elastin, collagen I, II, III
MT2-MMP	MMP-15	Gelatin, fibronectin, laminin, aggrecan
MT3-MMP	MMP-16	Gelatin, fibronectin, laminin
MT5-MMP	MMP-24	Gelatin, fibronectin, laminin
MT4-MMP	MMP-17	Gelatin, fibrinogen, fibrin
MT6-MMP	MMP-25	Gelatin
Macrophage elastase	MMP-12	Gelatin, elastin, fibronectin, laminin, collagen IV
-	MMP-19	Gelatin, aggrecan, elastin, fibrillin, collagen IV
Enamelysin	MMP-20	Gelatin, aggrecan
-	MMP-21	Aggrecan
CA-MMP	MMP-23	Gelatin, casein, fibronectin
-	MMP-27	-
Epilysin	MMP-28	-

Table 1.1 Human matrix metalloproteases (MMPs) and their matrix-associated substrates.

Information summarised from the findings of several researchers (Visse and Nagase 2003, Steffensen et al. 2001, Woessner 1991, Nagase et al. 2006).

1.2.4 Matrix metalloproteinases and physiological tissue repair

MMPs play a role in physiological wound repair as early as the first phase of haemostasis and inflammation. Infiltrating neutrophils at the wound site release MMP-8 (neutrophil collagenase), which is thought to be essential in physiological wound repair by regulating the inflammatory response (Gutiérrez-Fernández et al. 2007). MMP-8 plays a role in neutrophil migration through collagen degradation and the production of the chemotactic peptide Pro-Gly-Pro in a murine model of inflammation (Lin et al. 2008). Furthermore, the isolation of equine polymorphonuclear neutrophils (PMNs) from peripheral blood have been shown to be a strong source of MMP-9 (Clegg et al. 1997).

There is an abundance of evidence that demonstrates the release of growth factors from the ECM by a variety of MMPs. For instance, MMP-2 (gelatinase A), MMP-3 (stromelysin-1) and MMP-7 (matrilysin-1), have been shown to cleave a decorin-TGF-β1 complex into several fragments, releasing TGF-β1 *in vitro* (Imai et al. 1997). Further work in this area revealed that the release of TGF-β1 complexes from ECM occurred by the degradation of latent TGF-β by membrane type 1 matrix metalloprotease (MT1-MMP) (Tatti et al. 2008). Belotti and colleagues (2003) discovered a positive correlation with the amount of MMP-9 (pro-enzyme and active enzyme) and MMP-2 (pro-enzyme) and the release of the angiogenic growth factor vascular endothelial growth factor (VEGF) in a human-derived ovarian carcinoma xenograft mouse model. The authors then determined that MMP-9 and to a lesser extent MMP-2, increased levels of VEGF in the human ovarian carcinoma cell line SKOV3 *in vitro,* leading to endothelial cell motility. This cell motility was then fully inhibited by the VEGF inhibitor SU5416 (Belotti et al. 2003).

A study by Salo and colleagues (1994) demonstrated a role for MMP-9 in keratinocyte migration and granulation tissue formation in an experimental model of human oral mucosa wounding. The authors showed strong MMP-9 expression in the basal layer of the migrating epithelial sheet and granulation tissue up to 7 days post-wounding and a high expression of MMP-9 in the wound fluid of 2 and 4 day-old secretions post-wounding. The expression of MMP-2 remained constant in

fibroblasts alone (Salo et al. 1994). Subsequent studies by a different research group in 1998 supported the involvement of MMP-9 in re-epithelialisation, but also demonstrated collagenase and stromelysins-1 and -2 in populations of keratinocytes at the migrating wound edge (Madlener et al. 1998).

Various mouse studies have demonstrated the importance of MMPs and other proteases in wound closure. Plasminogen, a serine protease, was first demonstrated to play a role in wound healing when wound closure was significantly impaired in plasminogen deficient mice (Rømer et al. 1996). Lund and co-workers (1999) then went on to show the effects of the metalloprotease inhibitor galardin in wounded plasminogen-deficient mice, which resulted in delayed wound closure, demonstrating the overlapping functionality of MMPs and plasminogen in wound healing (Lund et al. 1999).

1.2.5 Protease levels in chronic wounds

Several studies have recorded elevated MMP levels in the wound fluid and tissues of chronic wounds. Yager and colleagues (1996) detected 10 to 25-fold increases in the gelatinases MMP-2 and MMP-9 in the wound fluid of patients with pressure ulcers when compared to healing wounds. Furthermore, collagenase was significantly increased in pressure ulcers compared to acute surgical wounds (Yager et al. 1996). The collagenase MMP-8 (neutrophil collagenase) has also been shown to be one of the predominant MMPs in both normal, healing wounds and non-healing chronic ulcers, with active forms of MMP-8 almost exclusively present in chronic wounds. Furthermore, reduced levels of the inhibitor TIMP-1 in chronic wounds and elevated MMP-8 has also been recorded (Nwomeh et al. 1999), demonstrating MMP:TIMP imbalances. Liu and colleagues (2009) showed an inverse correlation between MMP-9 and MMP9: TIMP-1 ratio to healing rates in the patients with diabetic foot ulcers, with the authors stating that increases in MMP-9 could be used to predict poor wound healing (Liu et al. 2009). Furthermore, various levels of the pro-enzyme state of the gelatinases MMP-2 and MMP-9 have been detected in the wound fluid of patients with chronic venous leg ulcers. In addition, the active enzyme form of MMP-

9 was highly expressed in wound fluid compared to wound tissues (Moor et al. 2009). Proteomic analysis of wound fluid from normal healing wounds and chronic leg ulcer wounds in diabetic patients revealed 188 differentially expressed proteins between the two wound types with regards to controlling inflammation, angiogenesis and cell motility. Of those differentially expressed proteins, more specifically MMPs and their inhibitors, MMP-1, MMP-2 and MMP-8 were shown to be up-regulated in chronic wounds. Furthermore, more frequent identification of peptides from the pro-peptide activation region of MMP-2 and MMP-9 were discovered in chronic wounds (Krisp et al. 2013).

1.3 Infection, Biofilms and Wound Healing

There are thought to be four states in which the presence of bacteria within a wound may be observed. The first state is the contamination of the wound area with the presence of non-proliferating bacteria on the superficial tissues. At this stage, this contamination is not thought to elicit a host immune response and does not affect wound closure. The second state, bacterial colonisation, involves the contamination of the wound area with proliferating bacteria, giving rise to the adherence of bacteria to superficial tissues and the formation of colonies. The presence of non-viable tissue within the wound provides an ideal environment for bacteria. At this stage, bacterial colonisation is not thought to cause a host immune response or affect wound closure. The third state is 'critical colonisation' and is a term coined to describe a delay in wound healing without clinical signs of inflammation. Here, bacteria have not managed to invade local tissues, however they are thought to secrete toxins and virulence factors that impair wound closure without eliciting an immune response. Critical colonisation is considered to be the point at which the wound can either improve following appropriate treatment, remain in a critical colonisation state, or deteriorate to clinical infection (White and Cutting 2006). However it is important to note that critical colonisation is indeed, a theory. The final state is known as bacterial infection and is characterised by the presence of proliferating bacteria that have invaded viable tissues and therefore initiate an immune response. Clinical

characteristics of bacterial infection include, tissue redness (erythema), pain, heat, swelling and excessive exudate at the site. This type of bacterial infection is considered to impede wound closure via several mechanisms including increased host protease and pro-inflammatory cytokine production and increased competition for oxygen and nutrients between both host cells and bacteria (Ovington 2003).

1.3.1 Biofilms

Microbial biofilms were recognised as early as the 1970's with the first observations describing natural populations of bacteria encased in a self-produced fibrous matrix within the slime located on the surface of mountain streams (Geesey et al. 1977). Biofilms can be described as communities of microorganisms that can attach to either abiotic (non-living surfaces such as metallic implants) or biotic surfaces (living surfaces such as human skin) or to each other, surrounding themselves in extracellular polysaccharide substances (EPS).

1.3.2 Biofilm development and intercellular communication

The development of a biofilm on a surface is thought to comprise of several different stages involving reversible and irreversible attachment, colonisation and dispersal (see **Figure 1.3**). The physiological processes of a biofilm such as the secretion of virulence factors or motility of individual microorganisms, is controlled by intercellular communication known as quorum sensing. Bacteria within the developing biofilm release chemical signals known as autoinducers, which are constitutively released from bacteria, therefore as the biofilm grows, the concentration of autoinducers increases. As the concentration of autoinducers reaches a critical threshold, a change in gene expression occurs, resulting in a physiological response, such as the release of virulence factors (Lindsay and Von Holy 2006, Mangwani et al. 2012). Both Gram-negative and Gram-positive bacteria secrete autoinducers. In brief, Gram-negative bacteria secrete acyl-homoserine lactones and Gram-positive bacteria release oligo-peptide molecules (Lindsay and

Von Holy 2006). The Gram-negative bacterium *P. aeruginosa* has been reported to secrete the quorum sensing molecule N-(3-oxo-dodecanoyl)-L-homoserine, which has been shown to enhance *P. aeruginosa* biofilm virulence and repress host immune responses (Driscoll et al. 2007). Indeed, *P. aeruginosa* has been implicated in a number of infectious diseases including chronic lung infections such as cystic fibrosis, thus research into the viability of targeting these quorum sensing molecules for drug development using quorum sensing inhibitors, has gained precedence (Hentzer et al. 2003).

1.3.3 Biofilm recalcitrance

Biofilms are thought to be responsible for a plethora of chronic infections including chronic lung infections, periodontitis, endocarditis, osteomyelitis and chronic wounds (Singh et al. 2000, Distel et al. 2002, Gristina et al. 1985a, James et al. 2008, Mohamed et al. 2004). Furthermore, biofilms have been associated with the use of biomaterials, implants and devices, from urinary catheters, intravenous lines and prosthetics, which can ultimately lead to systemic infection. The presence of biofilms in the healthcare setting was recognised as a potential impact to health from the mid-1980's when microorganisms encased in an extracellular matrix on the sutures of surgical wounds were identified (Gristina et al. 1985b). Subsequent to this, it appeared that bacteria growing within the biofilm state were more resistant to antibiotics and that biomaterial substrata acted as sites of colonisation and potentially facilitated bacterial virulence (Gristina et al. 1987).

There are many theories involving the potential mechanisms by which biofilms are considered able to confer resistance. It is thought that the presence of EPS that encases the microorganisms, results in the incomplete penetration of antimicrobials through the surface layers of the biofilm (Francolini and Donelli 2010). Another theory involves the slow growth rate within areas of the biofilm with reduced oxygen and nutrients, which is thought to hamper the actions of many antimicrobials that require a certain degree of cellular metabolic activity in order to function (Hall-Stoodley et al. 2004). Phenotypic variants that are considered to demonstrate a

highly resistant phenotype when compared to other microorganisms within the biofilm, commonly referred to as 'persister cells', confer resistance within the biofilm due to their slow rate of growth. Although these persister cells lack the genetic traits that resemble that of antibiotic resistance, they show high levels of multi-drug tolerance. (Hall-Stoodley et al. 2004, Spoering and Lewis 2001, Lewis 2008, Lewis 2010, Percival et al. 2011a).

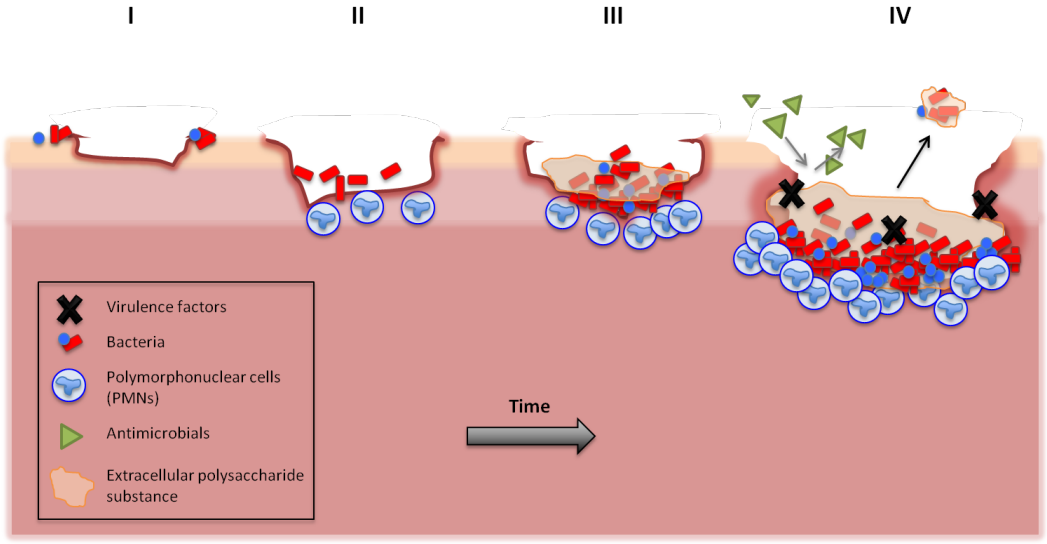


Figure 1.3 Schematic representation of biofilm development within a human wound.

I: Bacteria, either present on the skin or contamination from an external source, reversibly attach to areas of slough or dead tissue. **II**: Bacteria on the surface of the wound proliferate and become irreversibly attached through the use of bacterial appendages that anchor the bacteria to the tissue. **III**: Colonisation occurs when attached bacteria proliferate and produce extracellular polymeric substances (EPS), which is thought to protect the bacteria from external disruption. **IV**: The mature biofilm, surrounded in EPS, is resistant to antimicrobial therapy. The release of virulence factors helps protect from a host immune response. Parts of the mature biofilm can break away from the main biofilm, a process known as dispersal. Ultimately the dispersal of bacteria from the biofilm can lead to the dissemination, whereby these bacteria attach and colonise new sites, perpetuating infection.

1.3.4 Evidence of biofilms in chronic wounds

The investigation of biofilm-infected chronic wounds is an area of research that remains under much scrutiny and controversy. Probable reasoning behind this could be due to various chronic wound models; the way in which biofilms are grown in *in vitro* and *in vivo* models and the diversity of chronic wound sub-types.

The most commonly isolated bacteria in human chronic wounds include *Staphylococcus aureus* (93.5%), *Enterococcus faecalis* (71.1%), *P. aeruginosa* (52.2%) and coagulase negative *Staphylococci* (CoNS) (45.7%) (Gjødsbøl et al. 2006). Other research groups have extended this research in an attempt to gain a more comprehensive understanding of the microbial profile of wounds. The technique of 16S rDNA amplification followed by pyrosequencing, denaturing gradient gel electrophoresis (DGGE) and full ribosome shotgun sequencing has been used to determine the major microbial populations in diabetic foot ulcers, venous leg ulcers and pressure ulcers, whereby the most common wound isolates included *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia* and *Serratia* spp (Dowd et al. 2008). However it is interesting to note that the population profile of each type of chronic wound varied, with pressure ulcers housing more obligate anaerobes (62%) than both diabetic and venous ulcers (Dowd et al. 2008). Further research has shown *Streptococcus*, *Corynebacterium*, *Staphylococcus*, and *Pseudomonas* spp. to be among the most common primary genera of bacteria isolated from the pressure ulcers of 49 patients, which were identified using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) (Smith et al. 2010).

The presence of microorganisms within wounds has been recognised for some time; however the detection and identification of the microorganisms within a biofilm was under researched. In order to investigate this aspect further, James and colleagues examined for the presence of biofilms in both acute and chronic wounds, using both scanning electron microscopy (SEM) and polymerase chain reaction (PCR) coupled with DGGE. They discovered a significant difference in the presence of biofilms between chronic and acute wounds, with 60% of chronic wounds containing a

biofilm when compared to just 6% in acute wounds ($p<0.001$). Furthermore, PCR and DGGE revealed that these biofilms were polymicrobial (James et al. 2008). The presence of biofilms in chronic wounds has also been detected using peptide nucleic acid-based fluorescence *in situ* hybridisation (PNA-FISH), ultimately determining the structural organisation of bacteria within a chronic wound. The aggregation of bacteria into microcolonies within an alginate matrix with very few planktonic cells has been observed within chronic wounds (Kirketerp-Møller et al. 2008). In addition, a lack of correlation between the bacterial species identified using traditional culture techniques and PNA-FISH has been highlighted, with wound colonisation results showing >60% *S. aureus* and <30% *P. aeruginosa* using culture methods, and only 15% *S. aureus* and 70% *P. aeruginosa* using PNA-FISH (Kirketerp-Møller et al. 2008). This emphasises the importance of using not only traditional culture techniques, but also molecular methods, in order to achieve an accurate microbial identification and profiling of a wound.

1.3.5 Biofilms and host immune responses

A great deal of research has been dedicated to understanding host immunity in the control and tolerance of both commensal and pathogenic microorganisms. Despite this, a large portion of this research has been focussed upon invading bacteria within the single cell, planktonic state. It is now generally thought that the growth of microorganisms in both environmental and clinical settings is within the biofilm form. Thus research into the host immune response toward microorganisms within a biofilm has gained precedence.

The host's immune responses do indeed respond to the presence of biofilms and has been associated with the infiltration of neutrophils to the wound site in human chronic venous leg ulcers. Furthermore, the number of neutrophils in chronic venous leg ulcer biopsies containing *P. aeruginosa* was significantly higher than biopsies containing *S. aureus* (Fazli et al. 2011). Lactoferrin, a component of innate immunity that is abundant in surface secretions, was shown to stimulate twitching, a form of bacterial motility, in *P. aeruginosa* PA01, effectively preventing biofilm formation *in*

vitro (Singh et al. 2002). Cellular components of host immunity are not always effective in the prevention of biofilm formation. For example, the yeast *Candida albicans* has demonstrated successful biofilm formation in the presence of peripheral blood mononuclear cells (PBMCs) in an cytokine-rich environment (Chandra et al. 2007). Furthermore, the presence of polymorphonuclear leukocytes (PMNs) has been shown to enhance *P. aeruginosa* biofilm formation and development *in vitro* through the deposition of neutrophil-derived polymers, actin and DNA, acting as a scaffold for microbial colonisation and biofilm formation (Walker et al. 2005).

Biofilms have also demonstrated the ability to evade host immune responses. For instance, the EPS matrix of *P. aeruginosa* biofilms has been shown to protect against interferon-γ-(IFN-γ)-mediated macrophage killing (Leid et al. 2005). Additionally, *P. aeruginosa* biofilms have been shown to escape PMN-mediated phagocytosis through the secretion of a quorum sensing-regulated rhamnolipid, which effects PMN cell migration and causes the rapid, necrotic killing of PMNs *in vitro* and *in vivo* (Jensen et al. 2007). A study by Thurlow and colleagues (2011) investigated the innate immune responses directed towards a *S. aureus* biofilm in a mouse model of catheter-associated biofilm infection. The latter researchers found that the presence of the biofilm did not elicit a response from pattern recognition receptors such as Toll-like receptors (TLRs). Instead, significant reductions in the cytokines and chemokines IL-1β, tumour necrosis factor-alpha (TNF-α), CXCL2 and CCL2, and reduced macrophage infiltration into the biofilms were observed. *In vitro* co-culture of macrophages and *S. aureus* biofilms showed a limited phagocytosis response from macrophages, indicative of alternatively activated M2 macrophages, a more reparative phenotype when compared to the pro-inflammatory phenotype of M1 macrophages (Thurlow et al. 2011). The accumulation of evidence to support host immune evasion by microbial biofilms could be a conduit to disease pathogenesis in an immuno-compromised host.

1.3.6 Effect of biofilms on host protease expression

There is very little data on the effect of biofilms on host-protease expression as most *in vitro* and *in vivo* studies that have been undertaken focus on bacterial infection in the planktonic form. In a mouse model of *P. aeruginosa*-related corneal infection, there was a marked induction of MT-MMP-4, -5 and -6 (Dong et al. 2001). *Burkholderia cenocepacia* infection of an *in vitro* monolayer of lung epithelial cells resulted in the up-regulation of MMP-2 and MMP-9 genes and caused an increase in the secretion of active MMP-9 protein. This increase in active MMP-9 resulted in delayed wound closure in lung epithelial cells *in vitro*, which was reversed in the presence of a MMP-9-specific inhibitor (Wright et al. 2011).

The importance of investigating bacteria in planktonic and biofilm form in chronic wound pathogenesis has been recognised. Kirker and colleagues (2012) investigated the effect of methicillin-resistant *S. aureus* (MRSA) planktonic- and biofilm-conditioned medium on human dermal fibroblasts (HDFs) in a monolayer scratch wound model. Planktonic-conditioned medium (PCM) induced an increase in IL-6, IL-8, VEGF, TGF-β1, MMP-1 and MMP-3, whereas treatment with biofilm-conditioned medium (BCM) had a greater effect on the release of TNF-α when compared to PCM and a suppression of MMP-3 when compared to controls (Kirker et al. 2012). This study highlighted the differences in the soluble factors released by *S. aureus* in planktonic and biofilm form and that these two different physiological states elicit differential effects on the production of growth factors, cytokines and MMPs in host cells. Given that MMPs have been shown to be up-regulated in chronic wounds, the suppression of MMP-3 is unexpected and unexplained; therefore further investigation into the regulation of MMPs in response to biofilms is warranted.

1.4 Bacterial proteases

Bacterial proteases encompass a large and diverse group of proteases that are ubiquitously produced by all microorganisms and possess varying physiological and biochemical functions (Schaechter 2009). The intracellular expression and extracellular secretion of proteases in both Gram-positive and Gram-negative

bacteria are fundamental contributors to infection through the turnover of unfolded proteins in the host environment and the proteolysis of regulatory proteins upon environmental stimuli. The main intracellular conserved proteases include Lon, Clp, High temperature requirement A (HtrA) and FtsH. The biological functions of intracellular bacterial proteases and their role in infection has been extensively studied and reviewed elsewhere; the reader is directed to an excellent review by Ingmer and Brøndsted for a more in-depth account (Ingmer and Brøndsted 2009). This section of the introduction shall discuss what is known about the extracellular secreted proteases from the bacteria most commonly associated with infected chronic wounds, more specifically *S. aureus* and *P. aeruginosa*. Many species of bacteria are able to produce and secrete proteases, which can be toxins or virulence factors, whilst others may have a more central role in the degradation of proteins. Similar to proteases produced in humans, bacterial-derived proteases can also be categorised into metallo-, serine, aspartic and threonine proteases.

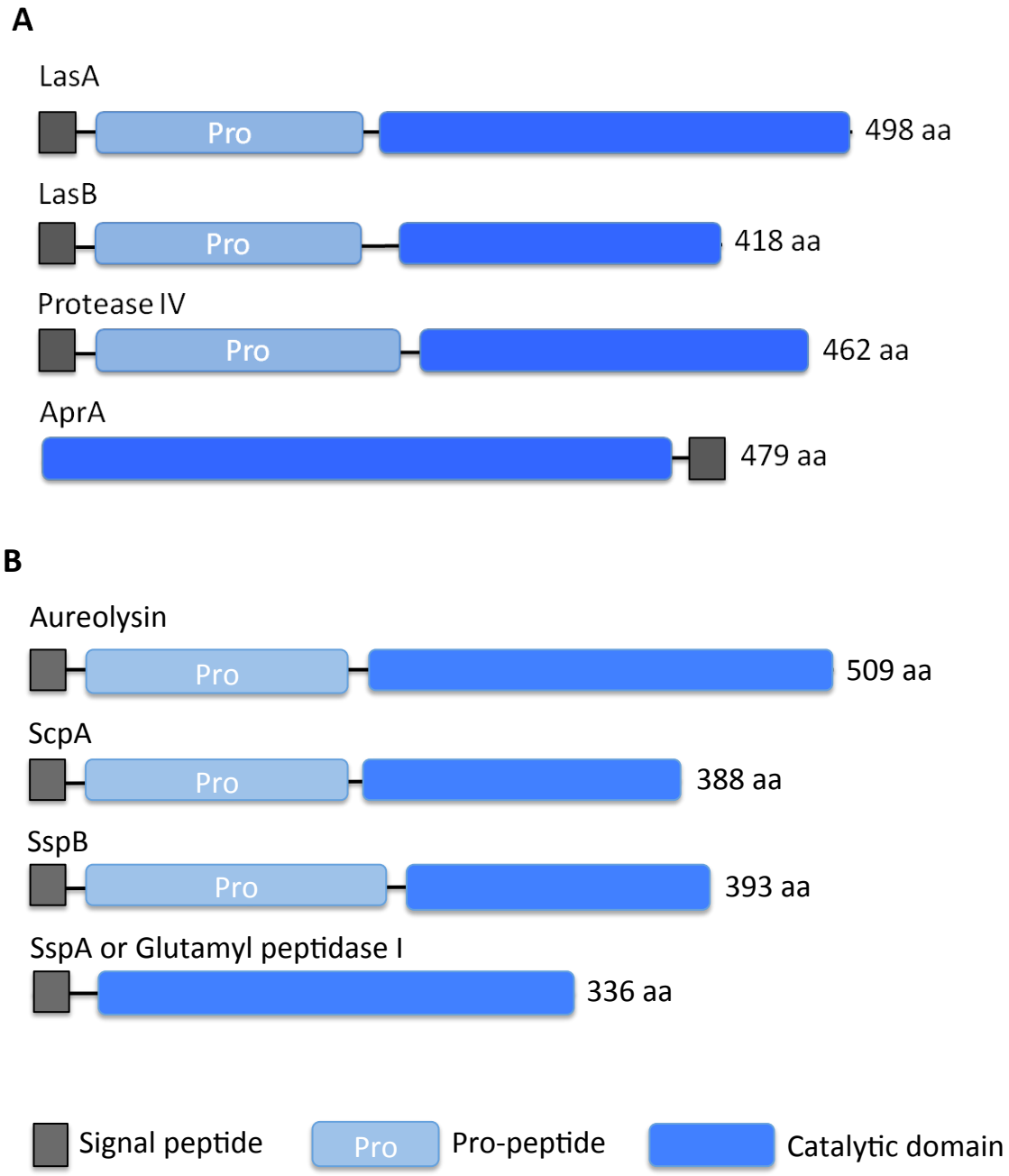


Figure 1.4 Schematic domain architecture of the major secreted proteases of (A) *P. aeruginosa* and (B) *S. aureus*.

Abbreviations include: amino acids (aa), elastase A (LasA), elastase B (LasB), alkaline protease (AprA), staphophain A (ScpA), staphopain B (SspB) and staphylococcal serine protease (SspA).

1.4.1 Extracellular metalloproteases secreted by P. aeruginosa

The MEROPS peptidase database contains information of a plethora of *P. aeruginosa* strains, with fully sequenced genomes for 14 strains, one of those including the commonly used laboratory strain *P. aeruginosa* PA01 (Rawlings et al. 2012). *P. aeruginosa* PA01 contains 5572 genes in its genome, of which 166 are identified as proteases. Of these proteases, the main proteases families include 62 serine-proteases, 45 metallo-proteases, 8 cysteine-proteases and 3 aspartic proteases. The most common *P. aeruginosa*-derived metalloproteases that have been shown to interact with the host during infection include elastase B (LasB), elastase A (LasA) and alkaline protease (AprA) (see **Table 1.2** and **Figure 1.4**).

1.4.2 P. aeruginosa secreted proteases: Molecular structure and synthesis

Elastase B (LasB) is a metalloprotease that is produced within the cell in a pre-proenzyme form, consisting of two pro-peptide domains and a third catalytic domain. The first 23 amino acids on the N-terminal codes for the signal peptide, which directs the translocation of the pro-enzyme across the inner membrane into the periplasm via the secretory system. This process results in the cleavage of the signal peptide. The unfolded, inactive pro-enzyme is then folded into its mature, active form by the pro-peptide domains, which act as a chaperone to assist correct protein folding. The mature LasB is then translocated across the outer membrane and the pro-peptide domain that has an inhibitory effect on the mature elastase B, is auto-catalytically cleaved and is degraded in the extracellular environment (Rawlings et al. 2012, Hoge et al. 2010).

Elastase A (LasA) is the most abundantly secreted metalloprotease of *P. aeruginosa*, containing a 31 amino acid coding N-terminal signal peptide, a pro-peptide domain and a catalytic domain. The synthesis and secretion of LasA in its mature form is similar to that of LasB. However pro-peptide cleavage is not auto-catalytic; instead it is cleaved by other proteases produced by *P. aeruginosa* (Hoge et al. 2010).

Alkaline protease (AprA) is a secreted metalloprotease, which in its inactive form, contains a single catalytic domain and a C-terminal signal peptide. In contrast to LasA and LasB, an alternative pathway controls the synthesis and secretion of AprA. The genetic locus associated with the synthesis and secretion of AprA contains five open reading frames that encode for AprA itself (aprA), a protease inhibitor (aprI) and the three proteins aprD, aprE and aprF, which are involved in the translocation of AprA across the inner and outer membrane of the cell (Duong et al. 1992).

1.4.3 P. aeruginosa-derived proteases in disease pathogenesis

The extracellular proteases that are produced by *P. aeruginosa* have been implicated in a range of pathologies. The pathophysiology of corneal ulcers for instance, has been characterised as the breakdown of extracellular matrix, and indeed, *P. aeruginosa*–derived extracellular proteases have been implicated in the development of corneal ulcers. Matsumoto and colleagues showed that the presence of *P. aeruginosa* 599A strain, isolated from a vitamin A-deficient rat eye, and *P. aeruginosa*-RPS, isolated from human corneal ulcer, resulted in the cleavage of the pro-MMP-2 to release the active MMP-2 from human corneal fibroblasts. The authors stated that the cleavage of pro-MMP-2 might have been due to the presence of 53kDa, 70kDa and 110kDa proteases, and 53kDa extracellular proteases from the *P. aeruginosa*-RPS and *P. aeruginosa* 599A strain respectively (Matsumoto et al. 1993). *P. aeruginosa* is a common bacterial pathogen identified in cystic fibrosis sufferers. There is evidence to suggest that bacterial serine proteases secreted by *P. aeruginosa* may affect the concentration of mucins within the mucus in the airways of patients with cystic fibrosis. Henke and colleagues reported a significant association with low mucin concentration and *P. aeruginosa* infection and increased degradation of mucins in *P. aeruginosa*-infected mucus. To test the hypothesis that *P. aeruginosa*-derived proteases degrade mucins, healthy mucus was incubated with synthetic human neutrophil elastase (HNE) and *Pseudomonas* LasB. This resulted in a significant degradation of mucins by the synthetic *Pseudomonas* LasB, which was comparable to degradation by HNE. Furthermore, the incubation of cystic fibrosis

sputum with the serine-protease inhibitors diisopropyl fluorophosphates (DFP), phenylmethylsulfonyl fluoride (PMSF) and 1-chloro-3-tosylamido-7-amino-2-heptanone HCl (TLCK), significantly reduced mucin degradation when compared to inhibitors of cysteine and metallo-proteases (Henke et al. 2011).

In the context of chronic wounds, there is some evidence to suggest that *P. aeruginosa*-derived metalloproteases may contribute to the pathogenesis of chronic wounds. One of the first research papers to acknowledge protease-producing bacteria in chronic wounds was published in 2001, whereby *P. aeruginosa* isolated from chronic leg ulcers showed varying levels of *P. aeruginosa* elastase, AprA and an unidentified 100kDa protease (Schmidtchen et al. 2001). Wysocki and colleagues identified 18 different bacterial species in chronic venous leg ulcers (10 Gram-positive and 8 Gram-negative) that displayed proteolytic activity. This proteolytic activity was not found to be consistent in any of these species after repeated isolation (Wysocki et al. 2012). However it is important to note that in this study, no quantitative data were presented to determine the varying levels of protease production between species, nor was there an attempt to identify these bacterial proteases. Another study by Wildeboer and colleagues sought to determine a correlation between protease activity and bacterial load in chronic wounds using fluorescent-labelled peptide substrates. Although there was no correlation between most species identified in the wound and protease activity, the signal detection of two substrates strongly correlated with *P. aeruginosa* numbers (Wildeboer et al. 2012). This study highlighted a potential use of these substrates as the basis for a diagnostic tool in the identification of *P. aeruginosa* colonisation in chronic wounds.

1.4.4 Extracellular proteases secreted by S. aureus

The MEROPS peptidase database contains fully sequenced genomes for 154 different subspecies and strains of *S. aureus* (Rawlings et al. 2012). In general, *S. aureus* produce 499 proteases, of which the major protease groups consist of 75 serine proteases, 169 metalloproteases, 84 cysteine proteases and 13 aspartic proteases respectively. The most commonly secreted proteases from *S. aureus* include the

metalloprotease aureolysin, the staphylococcal serine protease or Glutamyl peptidase I (SspA) and the cysteine proteases staphopain A (ScpA) and staphopain B (SspB) (see **Table 1.2**).

1.4.5 S. aureus secreted proteases: Molecular structure and synthesis

In its inactive form, the metalloprotease aureolysin consists of a 27 amino acid N-terminal signal peptide, a pro-peptide domain and a catalytic domain. Glutamyl peptidase I or SspA serine protease contains a 29 amino acid N-terminal signal peptide, pro-peptide doman and catalytic domain and is activated through its proteolytic cleavage by aureolysin. The cysteine protease ScpA contains a 25 amino acid N-terminal signal peptide, a pro-peptide domain and a catalytic domain in its inactive form. Post-translational modification of the ScpA protease into its active form is most likely through auto-catalytic processing. Similarly in its inactive form, the SspB protease has an N-terminal signal peptide (36 amino acids long), a pro-peptide domain and a catalytic domain. Upon secretion, the pro-enzyme is proteolytically cleaved by SspA (Rawlings et al. 2012).

1.4.6 S. aureus-derived extracellular proteases in disease pathogenesis

S. aureus extracellular proteases have been associated with virulence and persistent infection. Links between specific *S. aureus*-derived extracellular proteases and various types of infection has not received much attention. However a study by Zdzalik and co-workers investigated the prevalent *S. aureus* extracellular protease genes derived from cases of wound infection, pneumonia, sepsis, cystic fibrosis, skin infection and bone infection to name a few (Zdzalik et al. 2012). In this study they did not determine any correlation in gene expression patterns with specific types of infection, however they do state that most of the *S. aureus* proteases investigated, are expressed and secreted in the course of infection.

In order for a pathogen to successfully invade the host and cause persistent infection, the pathogen must be able to evade host immune responses. *S. aureus*

expresses and secretes proteases that have been shown to target components of host immunity such as the complement system. For example, the *S. aureus* metalloprotease aureolysin targets components of the immune system to facilitate immune evasion. Laarman and colleagues showed that the *S. aureus* aureolysin could effectively prevent complement-mediated phagocytosis through the cleavage of the C3 protein complex (Laarman et al. 2011). *S. aureus* cysteine proteases have also been implicated in the cleavage of a major protein in pulmonary surfactant, pulmonary surfactant protein A, which has been linked to a reduction in innate immune responses against lung infection such as neutrophil-driven phagocytosis (Kantyka et al. 2013).

1.5 Current Treatment Strategies for Chronic Wounds

The treatment of chronic wounds depends on the type of chronic wound and the severity of the wound. For example, pressure ulcers, in part, can arise from persistent mechanical pressure in those who have limited mobility and therefore using the technique of repositioning can help prevent the progression of this type of wound. Other treatment strategies such as negative pressure therapy (NPT), nutritional therapy and debridement are also used in the treatment of chronic wounds (see **Table 1.3**). However for all chronic wounds, the use of wound dressings is an integral part of wound care and can help manage excessive exudate and in some cases bacterial bioburden. A variety of wound dressings are used in the treatment of chronic wounds (see **Table 1.4**). Many of these dressings focus on the absorption of excessive wound exudate, whilst others combine absorptive and antimicrobial properties through the incorporation of ionic silver (Percival et al. 2011b). With regards to the regulation of excessive MMPs observed within chronic wounds, highly absorptive dressings and absorptive dressings with incorporated substrates such as collagen, have been shown to aid the sequestration of MMPs but also *Clostridium histolyticum* bacterial collagenase (Metzmacher et al. 2007).

1.6 Prospective Treatment Strategies

Potential future therapies to treat chronic wounds are on the horizon, with both *in vitro* and *in vivo* research developing new technologies to target biofilms, showing promising results.

1.6.1 Anti-biofilm technologies

With hypotheses surrounding the pathogenesis of chronic wounds relating to the involvement of biofilms, much research is now taking place to identify compounds with 'anti-biofilm' or biofilm-disrupting properties. Although there is a plethora of claims towards the anti-biofilm properties of many agents such as xylitol, lactoferrin and ethylenediaminetetraacetic acid (EDTA) *in vitro*, the evidence for their efficacy within *in vivo* models is currently lacking (Wolcott and Rhoads 2008, Ammons and Copié 2013). Nevertheless, the development and potential use of small molecules, bioactive molecules and enzymes provide promising opportunities for biofilm management in chronic wounds. Gawande and colleagues demonstrated the efficacy of a novel, naturally occurring enzyme-based gel on chronic wound–associated microorganisms (Gawande et al. 2014). Dispersin-B® -KSL-W gel containing the *Aggregatibacter actinomycetemcomitans*-derived enzyme Dispersin-B®, inhibits biofilm formation and disperses pre-formed biofilms. This gel showed both antimicrobial and anti-biofilm activity by significantly reducing log counts in chronic-wound associated MRSA, *S. epidermidis*, CoNS, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Gawande et al. 2014). Despite this, the dispersal of biofilms will ultimately lead to parts of the biofilm colonising new sites, resulting in the spread of infection. Therefore it is important that such technologies that aim to disrupt biofilm are combined with antimicrobials.

1.6.2 Quorum sensing disruption and inhibitors

Quorum sensing within bacterial biofilms is associated with biofilm development and enhanced virulence, making it an intriguing target for pharmacological action

(Hentzer et al. 2003). The proposed targets in the quorum sensing system include the signal generator, the quorum-sensing molecule and the signal receptor. In many cases, it is the signal receptor that is targeted by using molecules that block these receptors and disrupt signalling pathways (Rasmussen and Givskov 2006). Christensen and colleagues showed that *P. aeruginosa* biofilms in an *in vivo* mouse model could be disrupted by the use of the antibiotic tobramycin and several quorum-sensing molecules including furanone and horseradish juice extract. Synergy was seen between both treatments and the presence of quorum sensing inhibitor molecules, increased the susceptibility of the *P. aeruginosa* biofilm to tobramycin (Christensen et al. 2012). In the context of chronic wounds, Schierle and colleagues demonstrated reduced epithelialisation in a murine chronic wound animal model in the presence of *S. aureus* and *S. epidermidis* biofilm. Furthermore, the introduction of the quorum sensing disruptor RNAIII inhibiting peptide significantly restored re-epithelialisation when compared to the antibiotic oxacilin, due to reduced quantifiable bacterial burden. In addition, the staphylococcal biofilm signalling-associated gene, TRAP, appeared to play an important role in the reduction of wound closure, which was demonstrated by a weak effect on re-epithelialisation in $TRAP^-$ bacterial mutants (Schierle et al. 2009).

1.6.3 Photodynamic therapy (PDT)

Photodynamic therapy (PDT) is the application of a photoactive dye coupled with irradiation that brings about microbial cell death in the presence of oxygen. The anmicrobial effect of PDT has been well documented. The efficacy of this treatment in the eradication of biofilms has been investigated *in vitro* and *in vivo*. Di Poto and colleagues investigated the action of PDT and antibiotic treatment on the eradication of *S. aureus* biofilms *in vitro,* which resulted in a significant inactivation of *S. aureus* and when combined with the antibiotic vancomycin, showed an almost complete eradication of the bacteria (Di Poto et al. 2009). A small body of work exists for the treatment of biofilm-infected wounds in animal models. The use of PDT has shown a 98% reduction in *Escherichia coli* in excisional wounds of mice (Hamblin et al. 2002).

Further to this, *S. aureus*-infected leg wounds in mice were successfully treated using PDT, resulting in complete eradication of *S. aureus* in three of five mice (Gad et al. 2004). In humans, the treatment of two autoimmune ulcers in an Italian patient using PDT resulted in significant wound closure from 4.0cm to 1.8cm diameter. Given that these ulcers were previously shown to be highly recalcitrant to systemic antibiotics and the application of wound dressings; it is thought that the successful use of PDT could be through such mechanisms as enhanced keratinocyte migration, immunomodulatory activity and antimicrobial effectiveness (Motta and Monti 2007). PDT has been shown to significantly reduce bacterial load and complete wound closure in 50% of patients with chronic leg ulcers in a phase IIa, randomized, blinded and single treatment study (Morley et al. 2013). The use of PDT to reduce bacterial burden and encourage wound healing in chronic wounds is a promising potential therapy (Percival et al. 2014).

1.6.4 Bacterial proteases as therapeutic targets

The therapeutic targeting of proteases by pharmacologically attractive compounds has been successfully used in the treatment of many diseases, including hypertension, human immunodeficiency virus (HIV) and hepatitis C virus (HCV). For instance, pharmacologically approved serine protease inhibitor boceprevir (Victrelis; Merck) reversibly binds to and inhibits the HCV non-structural 3 (NS3) active site, preventing viral replication and thus sustaining the virologic response in patients with previously untreated, chronic HCV infection (Venkatraman 2012, Poordad et al. 2011).

The pharmacological targeting of bacterial proteases in the context of bacterial infection has not been fully exploited. Yet many of the secreted bacterial proteases are involved in bacterial virulence or growth and therefore the inhibition of these proteases may disrupt biofilm formation or increase susceptibility to antimicrobials (Kaman et al. 2014). In the context of chronic wound-associated bacteria, *P. aeruginosa* LasB protease has been investigated as a target of protease inhibition. A novel and potent inhibitor of LasB, *N*-mercaptoacetyl-Phe-Tyr-amide, has been

developed and shown to reduce *P. aeruginosa* biofilm growth, and when combined with additional antimicrobials such as ciprofloxacin and gentamycin, can fully eradicate the biofilm *in vitro* (Cathcart et al. 2011). Similarly, the deletion of LasB in *P. aeruginosa* PA01, referred to as a Lasb deletion mutant strain, has been shown to exhibit decreased bacterial attachment and microcolony formation. However microcolony formation in the LasB deletion mutants was restored following exogenous rhamnolipid supplementation, therefore it was hypothesised that LasB may promote biofilm formation through rhamnolipid-mediated regulation (Yu et al. 2014). The inhibition of other bacterial proteases however may not necessarily result in the disruption of the biofilm. Research by Loughran and colleagues identified that *S. aureus* aureolysin, and to a lesser extent, the proteases ScpA and SspB, actually promote the dispersal of *S. aureus* biofilms (Loughran et al. 2014).

Organism	Bacterial protease	MEROPS family	Protease	Substrate	Associated process	References
P. aeruginosa	Elastase A (LasA)	M23	Metallo-	Fibrinogen, elastin	ECM destruction	(Morihara et al. 1965, Rawlings et al. 2012)
	Elastase B (Las B)	M4	Metallo-	Elastin, col-III, col-IV, MMP-1/MMP-9 (pro-enzyme), elastase B	ECM destruction, MMP proteolysis, autoproteolytic processing	(Rawlings et al. 2012, Okamoto et al. 1997)
	Alkaline protease (AprA)	M10	Metallo-	Fibrinogen, gelatin, casein, haemoglobin, cytokines	Complement inactivation, host immune evasion	(Rawlings et al. 2012, Barrett et al. 2012, Parmely et al. 1990)
	Protease IV	S1	Serine-	Plasminogen, fibrinogen, complement protein C3	Complement inactivation	(Engel et al. 1998, Rawlings et al. 2012)
S. aureus	Aureolysin	M4	Metallo-	Plasminogen, complement protein C3	Complement inactivation	(Rawlings et al. 2012, Laarman et al. 2011, Massimi et al. 2002)
	Staphopain A (ScpA)	C47	Cysteine-	Elastin	ECM destruction	(Rawlings et al. 2012)
	Staphopain B (SspB)	C47	Cysteine-	Fibrinogen, fibronectin, elastin	ECM destruction, complement inactivation	(Rawlings et al. 2012, Massimi et al. 2002)
	Staphylococcal serine protease (SspA)	S1	Serine-	Actin, collagenase, IgG1 heavy chain, serum albumin, vimentin, casein	Host immune evasion, ECM degradation	(Rawlings et al. 2012, Massimi et al. 2002, Ryan et al. 2008)

Table 1.2 The major extracellular proteases associated with *P. aeruginosa* and *S. aureus*, their substrates specificities and associated biological processes.

Abbreviations include: extracellular matrix (ECM), matrix metalloprotease (MMP) and collagen (col).

Treatment	Description	Reference
Negative pressure therapy (NPT) or vacuum-assisted closure	NPT, whereby a vacuum is applied to a sealed wound dressing, is a method that has been shown to increase blood flow and reduce oedema, promoting granulation tissue formation and wound closure. There is conflicting evidence for the management of microbial bioburden.	(Levine et al. 2013, Argenta and Morykwas 1997, Mouës et al. 2011)
Repositioning	Particular method used in the management of pressure ulcers. Patients are repositioned to reduce mechanical stress on the chronic wound. For instance, adjusting the patient with a 30° tilt, three-hourly per night has been shown to reduce the incidence of pressure ulcers in the elderly.	(Moore et al. 2011)
Debridement	A method used to remove non-viable tissue or infected tissue to promote the development of healthy tissue. Methods of debridement: *Biological* - Larvae or maggots used to remove necrotic tissue; *Autolytic* - Naturally occurring enzymes breakdown dead tissue; *Chemical* - Use of chemical compounds such as hypochlorite; *Enzymatic* – Use of enzymes such as collagenase and papain; *Mechanical* - Mechanical debridement encompasses a variety implements from the use of wound dressings to the surgical removal of dead or contaminated tissue.	(Dryburgh et al. 2007, Wolcott et al. 2009)
Nutritional therapy	A change in the patient diet can be administered if malnutrition is suspected. Studies have shown that a higher calorie intake can reduce wound size.	(Ohura et al. 2011)

Table 1.3 Commonly used treatment options for chronic wounds.

Dressing	Description
Foam	An inert material with absorptive capacity and usually made from polyurethane.
Hydrocolloid	Dressing contains microgranules made from either natural or synthetic polymers such as gelatin, pectin or carboxymethylcellulose. The microgranules change to a gel-like state upon the absorption of wound exudate.
Hydrofiber	An absorptive fiber dressing that combines with wound exudate to transform into a hydrophilic gel.
Hydrogel	A water-based or glycerine-based hydrophilic polymer dressing. The dressing has absorptive properties but can also release water into dry, de-hydrated wounds.
Transparent film	A membrane-like film composed of polyurethane and polyethylene and coated with an acrylic adhesive.
Antimicrobial	**Description**
Chlorexidine	Antimicrobial activity again Gram-positive and Gram-negative bacteria.
Medical-grade honey	Natural, non-toxic antimicrobial.
Silver	Used for its broad-spectrum anmicrobial activity, it can be used topically or is quite often incorporated into composite wound dressings.
Sodium hypochlorite	Antimicrobial activity against fungi, viruses and vegetative bacteria.

Table 1.4 Commonly used wound dressings and associated antimicrobial incorporations in the management of chronic wounds.

Information taken from several sources (Lipsky and Hoey 2009, Mandal and Mandal 2011, Percival et al. 2011b, Zahedi et al. 2010).

1.7 Research Aims

The first research aim is to assess the proteolytic activity of *P. aeruginosa* and *S. aureus* isolated from equine and human chronic wounds and determine whether there is a difference in proteolytic activity between the planktonic and biofilm form of these isolates. The second research aim will then focus on the effect of planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM) from equine clinical isolates on the *in vitro* wound closure of normal fibroblasts (NFs) isolated from uninjured equine lower limb skin and granulation tissue fibroblasts (GTFs) cultured from equine chronic wound granulation tissue. The technique of zymography will be utilised to determine the release of host-derived MMPs in this wound model. In order to gain a better understanding of how infection affects MMP expression in host tissues, both normal skin and chronic wound granulation tissue from horse lower limbs will be assessed histologically for the presence of microorganisms within a biofilm, and the corresponding expression of MMPs in the surrounding tissue.

The next aim will then address the effect of PCM and BCM from human chronic wound-derived isolates on the *in vitro* wound closure of human dermal fibroblasts (HDFs) and human epidermal keratinocytes (HEKs). As with the equine model, host-MMP expression in response to PCM and BCM will be assessed. In addition, the generation of an *in vitro* 3-dimensional keratinocyte-fibroblast co-culture model will be developed, and wound repair observed in response to microbial bioburden.

I hypothesise that clinically derived isolates from both equine and human chronic wounds will secrete high levels of proteases. Furthermore, I believe that the presence of *S. aureus* and *P. aeruginosa* biofilms, but not planktonic bacteria, will reduce wound closure in equine and human models *in vitro* and will elicit the release of host-derived proteases. In addition I postulate that the identification of bacterial biofilms in the chronic wound granulation tissue of horses will correlate with a change in local MMP expression in surrounding tissues.

Chapter 2: Materials and Methods

2 Materials and Methods

2.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

2.2 Ethical Approval

Approval for the isolation of equine-derived bacteria was carried out in a previous study at the University of Liverpool and informed consent was obtained from the owners of the animals that participated in the study (Westgate et al. 2011). Human chronic-wound derived bacteria were a kind gift of Professor David Williams of Cardiff University and ethical approval for the isolation of these microorganisms was granted under their institution.

Approval for the harvest of equine skin from cadavers and equine granulation tissue following routine debridement for the isolation of fibroblasts was granted by the Ethics Committee, University of Liverpool and informed consent was obtained from the owners of the animals participating in this study.

2.3 Microbiology

2.3.1 Equine-derived Microorganisms

Equine-derived microorganisms were obtained from the University of Liverpool relating to the research undertaken by Westgate and colleagues (Westgate et al. 2011). Briefly, bacterial sampling in the form of a swabbing technique was performed on both acute and chronic equine wounds. Wound type was determined by the attending veterinary surgeon, whereby acute wounds were seen to progress at a normal rate and chronic wounds either showed little signs of healing or the presence of bright red colouration, discharge, malodour and exuberant granulation tissue. The

various microorganisms sampled from the wounds were identified using a variety of microbiological techniques, including selective agars, Gram-staining and tests to identify the production of oxidase, catalase and coagulase. All identified microorganisms were stored at -80°C in Microbank™ vials with cryopreservative and green-colour beads (Pro-lab Diagnostics, Cheshire UK).

2.3.2 Human-derived Microorganisms

Human chronic wound-derived bacterial isolates were a kind gift of Professor David Williams of Cardiff University. The isolation of these bacterial isolates was approved by a local research ethics committee and after informed consent was taken from patients attending the Wound Healing Research Unit, University Hospital of Wales (Cardiff, UK). Microbiological methods such as selective agars, Gram-staining and tests to identify the release of oxidase and catalase were used to identify the microorganisms.

2.3.3 Microbiological Culture

Prior to use, microorganisms were stored on Microbank™ green colour beads at -80°C before inoculating onto Mueller Hinton Agar (MHA) (Lab M, Bury, UK) and incubating overnight at 37°C. Following incubation, a loop of bacteria was transferred to Mueller Hinton Broth (MHB) (Lab M), Dulbecco's Modified Eagle Medium (DMEM) or EpiLife (Life Technologies, Paisley, UK) to obtain planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM) (see **2.3.4** and **2.3.5**). In general and for this study, the term planktonic was defined as bacterial cells that are grown in a free-floating, single-cell state. The term biofilm was described as bacterial cells that are attached to a surface and each other, and grow as a community of cells within a self-synthesised matrix. Growth curves of bacteria within a planktonic state was performed by inoculating MHB with the chosen microorganism and recording the absorbance of the inoculum at a range of time

points from 0-72 hours using the Thermo Scientific Multiscan FC plate-reader (Fisher Scientific, Loughborough, UK) at a wavelength of 600nm.

2.3.4 Bacterial planktonic-conditioned medium (PCM)

In order to obtain planktonic-conditioned medium (PCM), a loop of freshly cultured bacteria on MHA was used to inoculate either MHB, DMEM without phenol (Life Technologies, Paisley, UK) containing 10% Foetal Bovine Serum (FBS), 2mM L-glutamine (Life Technologies, Paisley, UK), 10mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (Life Technologies, Paisley, UK) and 0.5µg/ml Fungizone®Amphotericin B (Life Technologies, Paisley, UK) or Epilife medium containing human keratinocyte growth supplement (HKGS) (Life Technologies, Paisley, UK) before incubating overnight at 37°C. This specific preparation of DMEM is referred to as 'DMEM (without antibiotics)' throughout this thesis. Addtionally, this preparation of Epilife is referred to as 'EpiLife (without antibiotics)' throughout this thesis. Bacterial suspensions were diluted with either MHB or DMEM (without antibiotics) to approximately 10^9 colony forming units/ml, which was determined by measuring the optical density (OD) of the suspension to 0.4OD at 600nm using the Cecil CE 2040 spectrophotometer (Cambridge, UK). Once diluted, suspensions were cultured for 24 hours before being centrifuged at 11,000 x g at 4°C for 10 minutes in order to pellet the bacteria. Supernatants were obtained and sterile filtered using a Whatman 0.2µm syringe filter (Whatman Anodisc, Scientific Laboratory Supplies, Yorkshire, UK). Conditioned-medium was stored at -20°C until further use. PCM was used for either the scratch wound assays or general protease activity assays. In the scratch wound assays, PCM was diluted 1:1 with DMEM (without antibiotics).

2.3.5 Bacterial biofilm-conditioned medium (BCM)

For biofilm-conditioned medium (BCM), a loop of freshly cultured bacteria on MHA was used to inoculate DMEM (without antibiotics) before incubating overnight at 37°C. Cultures were then diluted to a concentration of 10^6 colony forming units/ml,

which was determined by using the Cecil CE 2040 spectrophotometer. Membrane filter discs of 0.2µm pore size (Whatman Anodisc, Scientific Laboratory Supplies, Yorkshire, UK) were sterilised using ultraviolet light exposure for 30 minutes before transferring the discs to MHA plates. Forty microliters of the bacterial suspension was added to the filter disc. The MHA plates containing the inoculated filter discs were incubated for 72 hours at 37°C in humidified conditions (see **Figure 2.1**). Following incubation, the discs were transferred to a Deep-Well plate (BD Biosciences, Oxford, UK). The wells of the plate were filled with 8ml of MHB or DMEM (without antibiotics), allowing the medium to touch the base of the filter disc. The Deep-Well plate containing the 72-hour biofilm was incubated at 37°C in humidified conditions for 24 hours. The conditioned-medium was collected after 24 hours and centrifuged at 11,000 x g at 4°C for 10 minutes. Supernatants were then sterile-filtered (0.2 µm) before being stored at -20°C until needed for further use. BCM was used in either scratch or general protease activity assays. In the scratch wound assays, BCM was diluted 1:1 with DMEM (without antibiotics).

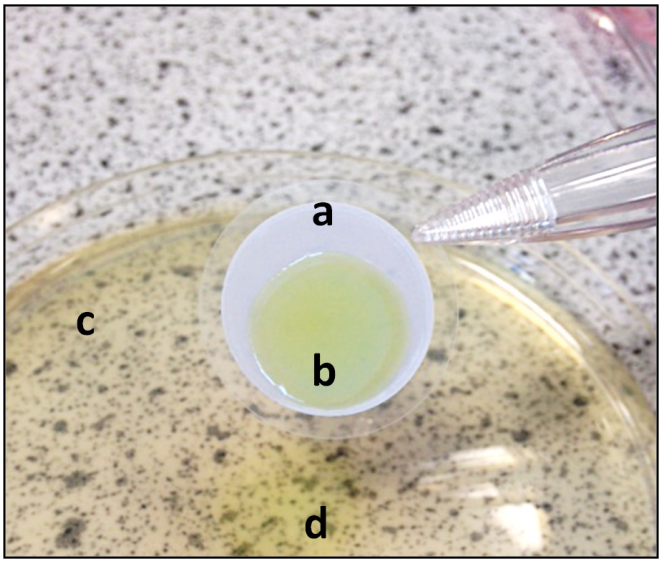


Figure 2.1 Representative image of a 72-hour human-derived clinical strain of *P. aeruginosa* grown as a biofilm.

The characteristic yellow/green *P. aeruginosa* biofilm (**b**) can be seen on the surface of the membrane filter disc (**a**). Mueller Hinton Agar (MHA) (**c**) was pigmented (**d**) from products released from *P. aeruginosa*, indicating exchange of nutrients and by-products through the membrane filter surface.

2.3.6 Crystal Violet Biofilm Forming Assay

To assess the biofilm forming potential of bacterial isolates, bacteria was cultured overnight in MHB at 37°C and diluted to 10^8 colony forming units/ml. Two-hundred microliters of the bacterial suspension in MHB was added to a 96-well microtitre plate before covering the plate with a 96-peg lid. The plate was incubated for either 24, 48 or 72 hours at 37°C with gentle shaking of 125rpm, allowing the bacteria within the suspension to attach to the pegs (see **Figure 2.2**). Following incubation, the pegs were removed from the bacterial suspension and were washed twice in phosphate buffered saline (PBS) and allowed to air-dry. The pegs were then incubated in a 0.5% crystal violet solution for 15 minutes at room temperature. The pegs were rinsed with distilled water, removing any excess crystal violet that had not specifically stained adherent bacterial cells. Excess distilled water was removed

before air-drying the pegs. The pegs were then immersed in 200µl of 30% acetic acid for 15 minutes, in a 96-well format, which solubilises the crystal violet stain. The pegs were removed from the 96-well plate and the absorbance of each well was read on a Thermo Scientific Multiscan FC plate reader (Fisher Scientific, Loughborough, UK) at a wavelength of 550nm. High absorbance values (strong crystal violet staining) were indicative of strong biofilm formation due to a greater number of adhered bacteria to the pegs. Similarly, low absorbance values and visually weak crystal violet staining corresponded to poor biofilm forming potential.

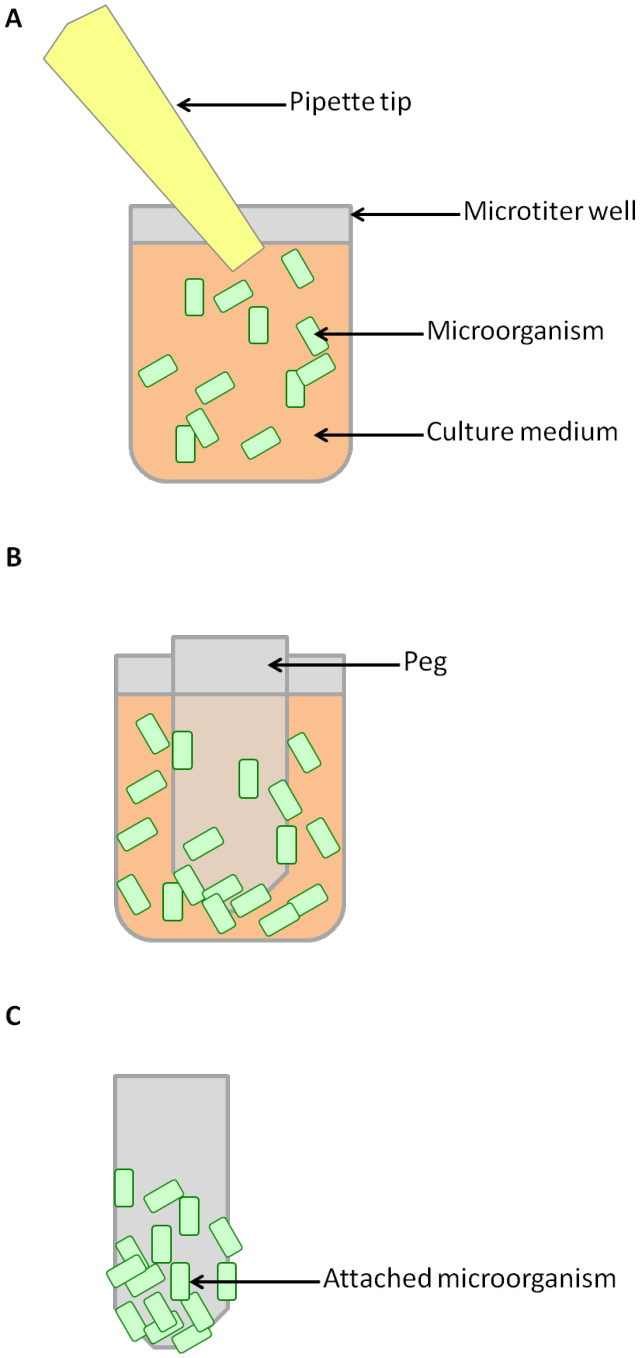


Figure 2.2 Schematic representation of biofilm formation on pegs.

(**A**) Microorganisms suspended in culture medium were added to the wells of a microtiter plate. (**B**) A microtiter lid containing plastic pegs was used to cover the plate, resulting in the submersion of the pegs into the microbial suspension. (**C**) Following incubation, the pegs were removed from the plate and washed. Microorganisms that were attached to the surface of the peg were termed a 'biofilm'.

2.4 Biofilm-disrupting technology study

The wound care company, and funders of this PhD, Advanced Medical Solutions Plc identified three potential biofilm-disrupting technologies, which were tested on three skin- and wound-related microorganisms, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *Candida albicans* ATCC 10321 (CultiLoops®, Remel, Thermo Scientific, US). The three potential biofilm-disrupting technologies included a chelating agent and two surfactants, surfactant A and surfactant B. The aim of this study was to identify whether these technologies could disrupt biofilms when combined with a commercially available polymer antimicrobial. More specifically, the minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) was determined. MBC was defined as the minimum concentration of antimicrobial that eradicates the growth of the dispersed, planktonic cells from the biofilm. MBEC was defined as the minimum concentration of antimicrobial that eradicates the biofilm.

2.4.1 Preparation of the biofilm-disrupting technologies

Dilutions of all biofilm-disrupting technologies were prepared using PBS. Stock solutions of each biofilm-disrupting technology were prepared, filter sterilised (0.2µm) and serially diluted in a 96-well plate format (see **Figure 2.3**). The first column of wells A1-H1 contained sterile MHB only to serve as no-growth controls, whilst the last column of wells A12-H12 contained MHB for growth controls. The highest concentration of biofilm-disrupting technology was added to the wells A11-H11 and was serially diluted in PBS across the plate, with the lowest concentration of biofilm-disrupting technology in wells A3-H3. The highest concentration of polymer antimicrobial was then added to wells H2-H11 and serially diluted in PBS up the plate, whereby the lowest concentration of polymer antimicrobial was in wells B2-B11. The resulting volume of each well in the treatment plate was 100µl; therefore each well containing the treatment, was diluted further by adding a 100µl of MHB. The checkerboard-style method allowed the determination of MBC and MBEC of

microorganisms after treatment with a variety of concentrations of both biofilm-disrupting technology and the polymer antimicrobial in combination and alone.

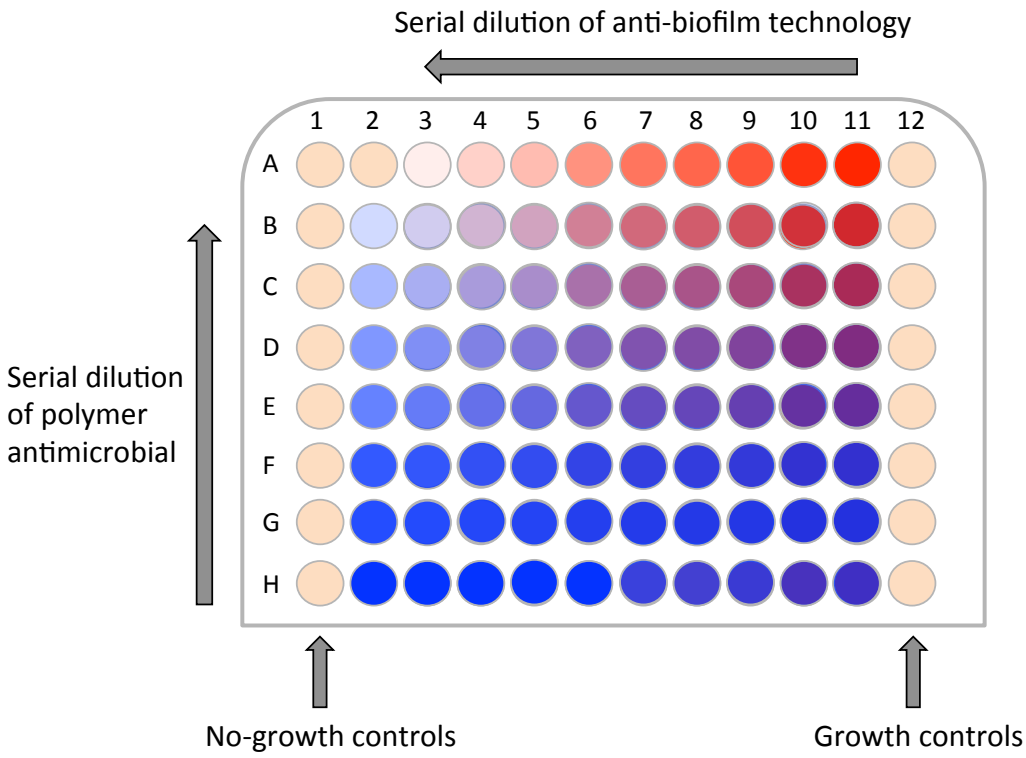


Figure 2.3 Treatment plate set-up containing serial dilutions of the anti-biofilm technology and polymer antimicrobial.

2.4.2 Determination of MBC and MBEC

Prior to use, microorganisms were stored on Microbank™ green colour beads at -80°C before inoculating onto MHA and incubating overnight at 37°C. Following incubation, a loop of bacteria was transferred to MHB and incubated overnight at 37°C. The microbial suspension was then diluted to 10^8 colony forming units/ml and

a volume of 180µl was transferred the wells of a 96-well plate. The plate was then covered with a 96-peg lid and incubated in a shaking incubator (125rpm) for either 24, 48 or 72 hours at 37°C. Biofilms grown on pegs for 24, 48 or 72 hours were then gently washed once in PBS before being transferred to the respective treatment plate and placed in a shaking incubator (125rpm) for 24 hours at 37°C. The 96-peg lid was subsequently removed from the treatment plate and transferred to a plate containing PBS (wash step). Each well of the treatment plate was then transferred to individual MHA plates and incubated at 37°C in order to detect microbial growth, which subsequently allowed the determination of the MBC. The 96-peg lid was then removed from PBS and transferred to a new 96-well plate containing 200µl of sterile MHB per well. The plate was then transferred to the Q-Series sonic bath (Ultrawave, Cardiff, UK) and sonicated for 20 to 30 minutes to remove adherent microorganisms from the pegs. The peg lid was finally removed and discarded. The contents of each well was transferred to individual MHA plates and incubated at 37°C in order to detect microbial growth, which subsequently allowed the determination of the MBEC. For no-growth controls, wells were filled with 180µl of MHB without bacteria.

2.5 Scanning Electron Microscopy

Bacterial biofilms grown on membrane filter discs were visualised using scanning electron microscopy (SEM). Stock solutions of 25% gluteraldehyde were diluted in PBS to a 2.5% working concentration. Bacteria grown for 72 hours were washed in PBS to remove non-adherent cells and fixed in 2.5% gluteraldehyde for 90 minutes at room temperature. Gluteraldehyde fixative was removed and the biofilms were washed twice in PBS. Biofilms were dehydrated in a series of ethanol concentrations ranging from 50% to 100%. Following dehydration the samples were sputter-coated using a Polaron E5100 sputter-coater with a gold/palladium target. Once coated, membrane filters containing the bacterial biofilms were attached to 25mm x 6mm aluminium stubs (Agar Scientific, Essex, UK) using two-sided sticky tape. Biofilms were imaged using the Hitachi TM3000 tabletop microscope (Daresbury Laboratories, Cheshire, UK).

2.6 Non-specific Protease Assays

2.6.1 Milk-Casein Agar

Milk-casein agar was used to assess the proteolytic activity of equine PCM at a preliminary level (see **Appendix I**). Milk-casein agar was prepared by adding 25g skimmed milk powder (BD, Oxford, UK), 2.5g casein (BDH, VWR, Lutterworth, UK), used for casein nutritional experiments, 1.25g yeast extract (Oxoid, Basingstoke, UK), 0.5g D-glucose (BDH, Oxford, UK) and 6.25g Number 1 agar (LabM Bury, UK) to 500ml distilled water. The agar was autoclaved, poured into petri dishes (approximately 30ml per plate) and allowed to cool until set. Milk-casein agar plates were dried in an oven to remove excess moisture before creating two 8mm wells in the agar, of equal distance apart, using a sterile punch biopsy. One hundred microlitres of bacterial supernatant was added to the first well followed by 100µl of MHB in the second to act as a negative growth and protease activity control. This was repeated for each bacterial supernatant before incubating the plates at 37°C for 24 or 48 hours. The experiment was performed three times using triplicate PCM. Plates were checked for proteolytic activity (zones of clearance) and measured at 24 hours and 48 hours (see **Figure 2.4**). The negative control (MHB) was not expected to show signs of activity with the milk-casein agar. If activity was present it was assumed that there was contamination of the MHB and the experiment was repeated with new PCM.

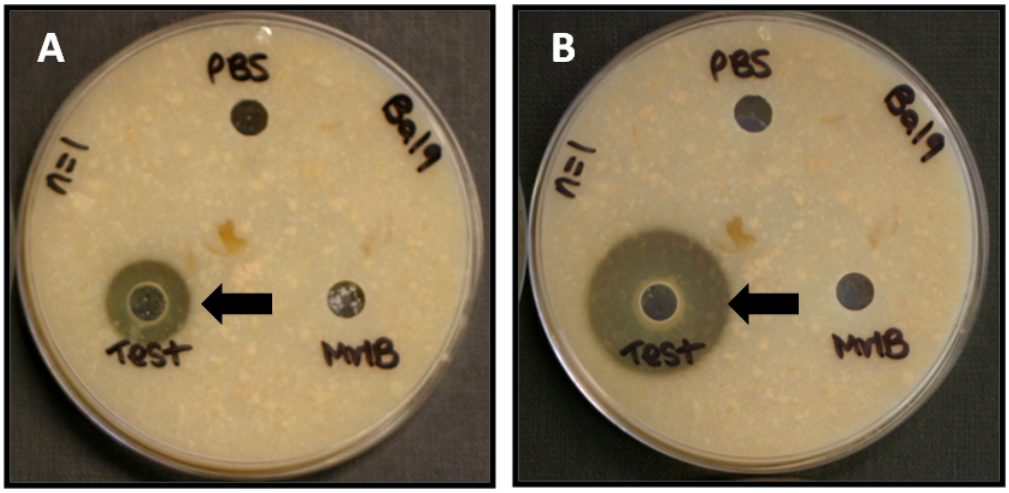


Figure 2.4 Zones of clearance in milk-casein agar.

Images of *P. aeruginosa* planktonic-conditioned medium (PCM) protease activity in milk-casein agar at 24- (**A**) and 48-hours (**B**) Black arrows indicate the zones of clearance whereby the casein has been degraded by proteases in the *P. aeruginosa* PCM. Images contain the abbreviations: Mueller Hinton broth (MHB), phosphate buffered saline (PBS).

2.6.2 Azo-Casein Assay

To measure the general protease activity of PCM and BCM, an assay utilising the chromogenic substrate Azo-casein, a non-specific protease substrate conjugated to an azo-dye, was performed. Briefly, Azo-casein solution was made by dissolving Azo-casein in distilled water to a concentration of 5mg/ml. Fresh Azo-casein was prepared for every new assay. Blank samples were made using 50µl medium, 50µl Azo-casein solution and 500µl PBS. Test samples were made using 50µl bacterial-conditioned medium, 50µl Azo-casein solution and 500µl PBS. All samples were tested in duplicate and incubated overnight at 37°C. Following incubation, 600µl of ice cold 5% trichloroacetic acid (TCA) was added to each sample and incubated on ice for 30 minutes. All of the samples were centrifuged at 11,000 x g at 4°C for 10 minutes. After centrifugation, 200µl of each sample supernatant was transferred to a

96-well plate before reading the absorbance on a Thermo Scientific Multiscan FC plate reader at a wavelength of 405nm. Absorbance values for duplicate blank samples were subtracted from test samples to give 'blank subtraction values'. The average of the blank subtraction values was calculated.

2.6.3 Azocoll Assay

To further assess the general proteolytic activity of PCM and BCM, an assay using a different non-specific substrate named Azocoll, a chromogenic azo dye-impregnated collagen, was performed (see **Figure 2.5**). Azocoll solution was made by adding Azocoll to a buffer containing 0.05M Tris-Hydrochloride (Tris-Hcl) and 1mM calcium chloride ($CaCl_2$), at a concentration of 5mg/ml. Fresh Azocoll solution was prepared for each new assay. Blank samples consisted of 50µl buffer and 90µl Azocoll solution. Test samples were made using 50µl bacterial-conditioned medium (PCM or BCM) and 90µl Azocoll solution. All samples were run in duplicate per assay and all assays repeated three times using triplicate bacterial-conditioned medium. Samples were incubated in a shaker (200rpm) overnight at 37°C. Following incubation, all samples were centrifuged at 10,000 x g for 8 minutes at 4°C. Sample supernatants were transferred to a 96-well plate and the absorbance read at 550nm on a Thermo Scientific Multiscan FC plate reader. Absorbance values for duplicate blank samples were subtracted from test samples to give 'blank subtraction values'. Averages of all blank subtraction values were taken of all duplicate test samples.

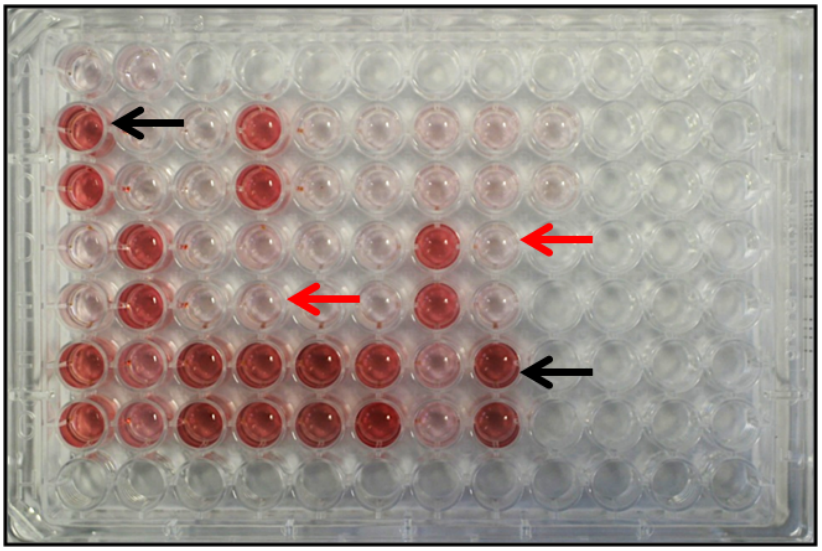


Figure 2.5 Azocoll assay

Image shows a microtiter plate with wells containing bacterial-conditioned medium and the Azocoll solution after incubation at 37°C. The presence of proteases in the bacterial-conditioned medium causes the hydrolysis of the collagen substrate, which releases the azo-dye. Black arrows indicate strong proteolytic activity, which appears a dark red colour. Red arrows indicate weak proteolytic activity, which appears clear/pale pink in colour.

2.7 Gel Electrophoresis

2.7.1 Substrate Zymography

A Miniprotean II gel system (Bio-Rad, Hemel Hempstead, UK) was employed for zymography gels. Samples were prepared 1:1:3 bacterial-conditioned medium (PCM or BCM) : Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) : distilled water and incubated at 37°C for 1 hour. A resolving gel was prepared using 30% acrylamide stock (Severn Biotech Limited, Kidderminster, UK) resolving buffer (1.5M Tris-Hcl, pH 8.8), 1% gelatin solution (EIA grade reagent gelatin) (Bio-Rad, Hemel Hempstead, UK) or 1% collagen solution (Type I from rat tail), distilled water, 20% sodium dodecyl sulfate (SDS), ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). The 0.25% gelatin and 7.5% polyacrylamide gel was cast into 1mm thick glass plates.

A small volume of isobutanol was laid over the gel to produce a level surface. Once the gel was set the isobutanol was washed away with distilled water and the stacking gel was cast. A 4% stacking gel was produced using 30% acrylamide stock, stacking buffer (0.5M Tris-Hcl, pH 6.8), distilled water, SDS, APS and TEMED. Once the stacking gel was added to the plates, a 10-well comb was inserted and the gel was allowed to set. Once set, the comb was removed and the plates were transferred to a gel running tank and filled with running buffer (concentrated 10x – 18g Tris base, 86.4g Glycine, 6g SDS and made up to 600ml with distilled water – diluted 1:10 for 1x). Incubated samples were loaded at a volume of 20µl per well. Gel electrophoresis occurred at a constant 200V until the sample bands were run off the bottom of the gel. The gel was then transferred to 2.5% Triton-X 100 for one hour to remove traces of the SDS. Following incubation the gel was washed thoroughly in distilled water before being placed in 1x refolding buffer (concentrated 10x – 6.05g Tris-base, 11.68g sodium chloride, 5.50g calcium chloride, 670µl Brij, pH 7.6) and incubated overnight at 37°C. Following incubation, the gel was removed from the refolding buffer and stained in 0.5% Coomassie Brilliant Blue G250 for 40 minutes. Stained gels were de-stained in a de-staining solution (150ml methanol, 35ml glacial acetic acid and 315ml distilled water), changing the de-stain solution every 15 minutes until clear bands were visible on a blue background. Following de-staining, bands representing enzymatic activity were present as clear bands on a dark blue background. To determine an approximate molecular weight for the protease bands, 7µl of Novex® Sharp Pre-stained Protein Standards (Life Technologies, Paisley, UK) were loaded onto all gels. Gels were visualised on the Syngene Gene genius Bio-imaging system using GeneSnap software.

2.7.2 Inhibitor Studies

Protease class was determined using inhibitors for cysteine, metallo and serine proteases. Zymograms were inhibited with 10mM N-ethylmalemide (NEM), 100mM EDTA, 50mM Benzamidine hydrochloride hydrate and incubated in normal refolding buffer as a control and refolding buffer supplemented with the inhibitor. Gels were

stained in 0.5% Coomassie Brilliant Blue G250 for 40 minutes as previously described (see **2.7.1**).

2.7.3 SDS PAGE

A 15% Acrylamide gel (resolving gel) was made and cast using 7.5ml 30% acrylamide/bis-acrylamide, 3.5ml distilled water, 3.75ml resolving buffer (1.5M Tris-HCL, pH8.8), 150µl 10% SDS, 75µl 10% APS and 10µl TEMED. Once the gel was added to the 1mm glass plates, a small amount of isobutanol was added to the gel to provide an even surface. Once the gel had set, the isobutanol was removed and the gel was washed with distilled water. A stacking gel was made and cast using 0.67ml acrylamide/bis-acrylamide, 3ml distilled water, 1.25ml stacking buffer (0.5M Tris-HCL, pH 6.8), 50µl 10% SDS, 50µl 10% APS and 20µl TEMED before inserting a 10-well comb. Test samples were prepared by diluting bacterial-conditioned medium in 1:1 in 2x Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) containing dithiothreitol (DTT) and heated for 10 minutes at 100°C. When the gels were set, the comb was removed and the wells washed thoroughly in distilled water. Gels were transferred to the gel tank and filled with NuPAGE MES SDS running buffer (Life Technologies, Paisley, UK) (1x) before loading the samples into the wells of the gel at 20µl per well. A Novex Sharp Unstained Protein Standard (Life Technologies, Paisley, UK) (7µl) was added to one of the wells to determine the molecular weight of proteins within the samples. Electrophoresis occurred at 150V until the dye front reached the bottom of the gel. The gels were removed and placed in 0.5% Coomassie Brilliant G250 overnight at room temperature. Following incubation, the gels were de-stained in 150ml methanol, 35ml glacial acetic acid and 315ml of distilled water, until blue bands appeared against a clear background.

2.8 Ammonium Sulfate Precipitation and Partial Purification of Proteins

Initially, to assess at which percentage of Ammonium Sulfate solid (AS) was needed to precipitate the target protease(s), 15-70% AS was tested and it was subsequently

found that 55% AS was sufficient to precipitate the target protease(s). Depending upon the working temperature and volume of the protein solution, the amount of AS solid to be added to the protein solution can be calculated from an AS precipitation table. In brief, BCM was transferred to a 50ml glass beaker on ice. AS was added slowly to the BCM whilst gently mixing so that the solution didn't become unevenly saturated with AS. After the addition of AS to the BCM, the solution was allowed to equilibrate for 20 minutes on ice. The solution was then transferred to a centrifuge tube centrifuged at 10,000 x g for 15 minutes at 4°C. After centrifugation, a pellet was observed, which contained the AS and the precipitated protein fraction. The supernatant was removed and the pellet was re-suspended in PBS (10% of starting volume). The re-suspended pellet was then transferred to dialysis tubing and placed in PBS overnight at 4°C to remove the AS. The protein concentration, content and proteolytic activity of the solution was then assessed using the BCA assay, SDS-PAGE, non-specific proteases assay and zymography. This solution was referred to as a partially purified protease. Partially purified protease was obtained from three *P. aeruginosa* biological replicates (P1097, P1140 and P1145). These proteases were then used in the human adult dermal fibroblast (HDF) and human adult epidermal keratinocyte (HEK) scratch wound assays (see **2.13**).

2.9 BCA Assay and Sample Protein Quantification.

The detection and quantification of total protein in the partially purified protease solution was performed using the Pierce® BCA Protein Assay Kit (Fisher Scientific UK Ltd, Loughborough, UK) to the manufacturer's instructions. Bovine serum albumin (BSA) was diluted in PBS to create BSA standard concentrations between 0mg/ml to 2mg/ml. The partially purified protease sample was diluted in BCA working reagent. BSA standards and test samples were performed in duplicate. The assay was incubated for 30 minutes at 37°C before the absorbance was read on the Thermo Scientific Multiscan FC plate reader at a wavelength of 570nm. Average absorbance values of the BSA standards were plotted against the known concentration to produce a standard curve. The equation of the standard curve was used to

determine the concentration of protein per sample based on the average absorbance values. Protein concentration of the partially purified protease was expressed as mg/ml.

2.10 Cell Culture

2.10.1 Equine fibroblasts

Equine normal fibroblasts (NFs) were obtained from cadavers (see **Figure 2.6**). Hair was removed using hair clippers and the surface of the skin was washed with Hibiscrub (containing 4% Chlorhexidine Gluconate) (MediSupplies, Poole, UK) to remove any microorganisms that may have caused infections in subsequent tissue culture. A small section of the skin was removed and dissected into small pieces (approximately 2mm x 2mm) using a scalpel and washed in HBSS. The tissue was then added to a $25cm^2$ tissue culture flask containing DMEM (with phenol red) (Life Technologies, Paisley, UK) supplemented with 100 units/ml penicillin-streptomycin (Life Technologies, Paisley, UK), 0.5µg/ml Fungizone®Amphotericin B (Life Technologies, Paisley, UK), 10mM HEPES (Life Technologies, Paisley, UK) and 10% FBS. This formulation of DMEM culture medium used to culture mammalian cells is referred to as DMEM (complete) throughout this thesis. For the initial culture of equine tissue, DMEM (complete) contained an additional 10ng/ml gentamicin (Life Technologies, Paisley, UK). The tissue was incubated in humidified conditions in 5% CO_2 at 37°C for 5 days before changing the medium. Following this incubation step, the tissue culture flask was microscopically checked on a daily basis for the presence of adherent fibroblasts. When adherent fibroblasts were observed, the tissue explants were removed and fibroblasts were cultured to 90% confluence before passage. Adherent cells were removed from the tissue culture flask using 0.05% Trypsin/EDTA (Life Technologies, Paisley, UK). The monolayer was washed twice in Hanks balanced salt solution (HBSS) without calcium and magnesium (Life Technologies, Paisley, UK) before adding Trypsin/EDTA to the tissue culture flask (just enough to coat the cells) and incubating at 37°C for 5 minutes. Once lifted from the flask, the cells were centrifuged at 1000 x g for 4 minutes and re-suspended in

DMEM (complete). Fibroblasts from three donors were used for scratch assays. Equine normal fibroblasts were used in experiments between passages three and five.

Equine granulation tissue fibroblasts (GTFs) were obtained from chronic wounds that were being debrided as part of routine clinical treatment to remove excessive granulation tissue. This involved sharp dissection and removal of granulation tissue raised above the level of the epithelial margin. The debrided tissue was treated similarly to normal skin, as described above, for fibroblast isolation. After five days in culture, the medium was changed, explants removed and GTFs were cultured to approximately 90% confluence before being sub-cultured and used for scratch wound assays. Three donors were used for the scratch wound assays and GTFs were used between passages three and five.

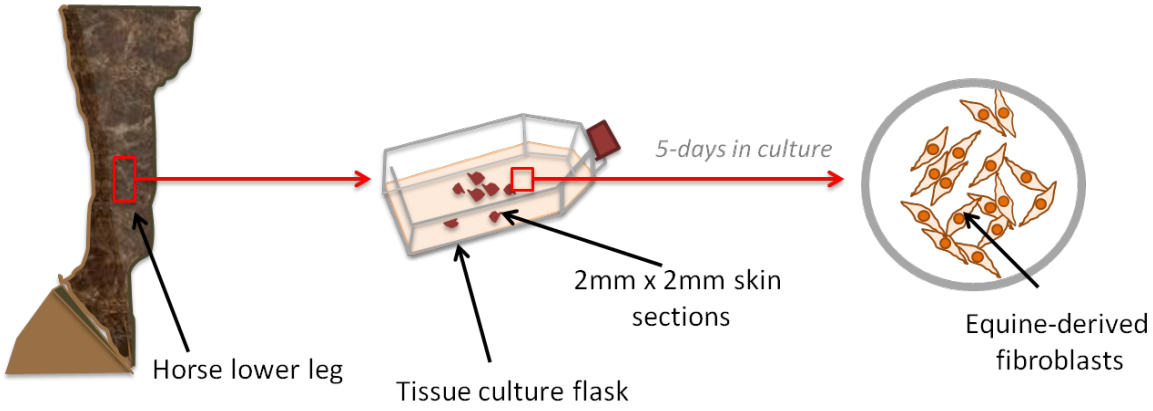


Figure 2.6 Schematic representation of the collection of equine tissue for tissue culture.

Normal skin from the lower leg of cadavers or granulation tissue from chronic wounds located on the lower leg was obtained using a scalpel. Tissue was cut into 2mm x 2mm pieces and transferred to a tissue culture flask containing DMEM supplemented with penicillin-streptomycin, Fungizone®Amphotericin B, HEPES and FBS. The tissue pieces were cultured for 5 days in 5% CO_2 at 37°C before changing the culture medium. Following incubation culture medium was changed and fibroblasts that had migrated out of the tissue could be identified microscopically.

2.10.2 Adult human dermal fibroblasts (HDFs)

Adult human dermal fibroblasts (HDFs) (Life Technologies, Paisley, UK) were cultured in DMEM (with phenol red) (Life Technologies, Paisley, UK) supplemented with 10% FBS, 100 units/ml penicillin-streptomycin (Life Technologies, Paisley, UK), 0.5µg/ml Fungizone®Amphotericin B (Life Technologies, Paisley, UK) and 10mM HEPES buffer (Life Technologies, Paisley, UK), also referred to as DMEM (complete). Cells were cultured until approximately 90% confluence before passage. HDFs were used in scratch wounds and to create the dermal layer of the skin construct. HDFs cells were used between passages four and seven.

2.10.3 Adult human epidermal keratinocytes (HEKs)

Adult human epidermal keratinocytes (HEKs) (Life Technologies, Paisley, UK) were cultured in EpiLife medium (Life Technologies, Paisley, UK) supplemented with human keratinocyte growth supplement (HKGS), 100 units/ml penicillin-streptomycin (Life Technologies, Paisley, UK), 0.5µg/ml Fungizone®Amphotericin B (Life Technologies, Paisley, UK). HEKs were cultured until 80% confluence before passage. HEKs were used to create the epithelial layer of the skin construct and were used between passages three and six. All of the cells used in the skin constructs were cultured in humidified conditions with 5% CO_2 at 37°C.

2.11 MMP-2 isolation

To provide a positive control for substrate zymography and also to assess the efficacy of MMP sequestration by a variety of wound dressings (see method **2.11** and **Appendix II**), the isolation and purification of MMP-2 from equine normal fibroblasts (NFs) was carried out. The isolation of NFs was carried out as described earlier in this chapter (see **2.9.1**). NFs were cultured in T175 tissue culture flasks for the optimal volume of crude MMP-2. When the NFs were approximately 90-95% confluent, DMEM (complete) was removed from the flask and the cells were washed in HBSS (without calcium and magnesium) in order to remove traces of FBS. The cells were

then treated with serum-free DMEM containing 100 units/ml penicillin-streptomycin, 0.5µg/ml Fungizone®Amphotericin B and 10mM HEPES buffer (approximately 30-35ml in a T175 tissue culture flask) and incubated in 5% CO_2 at 37°C for 5 days. Following incubation, the NF-conditioned medium was collected and centrifuged at 1000 x g for 4 minutes to remove cells. The NFs that remained in the tissue culture flask were disposed of. Conditioned medium containing crude-MMP-2 was then stored at -20°C until ready for enzyme purification.

2.11.1 MMP-2 purification using gelatin sepharose chromatography

The gelatin sepharose chromatography column was prepared by placing a small amount of glass wool in the bottom of a 20ml syringe (without needle) before adding 5ml of gelatin sepharose (GE Healthcare, Little Chalfont, UK) to the surface of the wool. The column was washed twice in equilibration buffer (0.05M Tris Base, 0.5M $NaCl_2$, 0.005M $CaCl_2$, 0.05% Brij, 0.02% sodium azide, pH 7.6) before adding the cell supernatant (crude MMP-2) to the column and allowing the supernatant to pass through the column into a collection tube. The collected supernatant was discarded. The column was washed four times using the equilibration buffer (5ml each wash). At this stage, the MMP-2 was bound to the gelatin sepharose. The MMP-2 protease was then eluted from the column using 10ml of 10% DMSO in 80% elution buffer (0.05M Tris HCl, 1M $NaCl_2$, 0.005M $CaCl_2$, 0.05% Brij, 0.02% sodium azide, pH 7.6), which was collected in 15ml centrifuge tubes. The eluted MMP-2 was then transferred to dialysis tubing, which was incubated in MMP dialysis buffer (0.05M Tris HCl, 0.005M $CaCl_2$, 0.05% Brij 35, 0.02% sodium azide, pH 7.6) at 4°C overnight. Post-dialysis, MMP-2 was stored in aliquots at -20°C until needed. The gelatin sepharose column was washed four times in equilibration buffer (5ml each wash) before adding 5ml equilibration buffer to the column and storing at 4°C in a sealed bag to prevent dehydration.

2.12 Wound dressing studies: Sequestration of MMP-2

It was proposed by Advanced Medical Solutions Plc to test the efficacy of several wound dressings to sequester proteases, specifically MMP-2. As the overall focus of this thesis concerned bacterial biofilms and their extracellular proteases, the results of this study can be found in Appendix II of this thesis. In brief, 0.01g of each dressing sample was individually incubated with 250µl of purified MMP-2, in a 12-well plate format, for 1, 3, 6, 24 or 48 hours at 37°C. After the specified incubation period, 500µl of sterile PBS was added to the dressings and the supernatant was collected and stored at -20°C until further use. Samples were then assessed for MMP-2 activity using substrate zymography (see method **2.7.1**).

2.13 Scratch wound assay

The *in vitro* scratch wound model is a commonly used model to assess cell migration and wound closure due to its simplicity, reproducibility and cost effectiveness (Liang et al. 2007). Therefore this model was used to assess the wound closure of various cell types in response to bacterial-conditioned medium. Furthermore the model was used to assess the release of host proteases and in the case of HDFs, the expression of MMP-1, -2, -9, -13 and collagen-I at a transcriptional level in response to bacterial-conditioned medium. HEKs, HDFs, NFs or GTFs were seeded into 6-well plates at a cell density of 1 x 10^5 cells/ml in EpiLife or DMEM (complete). Viable cell numbers were determined using trypan blue exclusion dye. Cells were cultured until 95% confluence before creating a scratch using a sterile 100µl pipette tip. Cells were washed using PBS to remove cell debris caused from the scratch. Bacterial-conditioned medium, both PCM and BCM, were diluted 1:1 with DMEM (without antibiotics). Two millilitres of the diluted bacterial-conditioned medium was added per well. Both untreated unscratched and scratched monolayers were included in this assay as controls. The treated and untreated cells were incubated in atmospheric, humidified conditions at 37°C with 5% CO_2 for 48 hours (see **Figure 2.7**). Images of the scratch area were taken at 0, 24 and 48 hours. To ensure images were

taken in the same place each time, a line was drawn on the underside of the dish, perpendicular to the scratch. Images were taken using the Nikon Eclipse microscope (Melville, USA) using x10 magnification. Aliquots of media (100 µl) were taken from each well at 0, 24 and 48 hours to determine the presence of proteases using gelatin zymography. At 48 hours, the cells were lysed in 1 ml of TriZol reagent (Life Technologies, Paisley, UK) per well and stored at -80°C in preparation for qRT-PCR (see **2.16**).

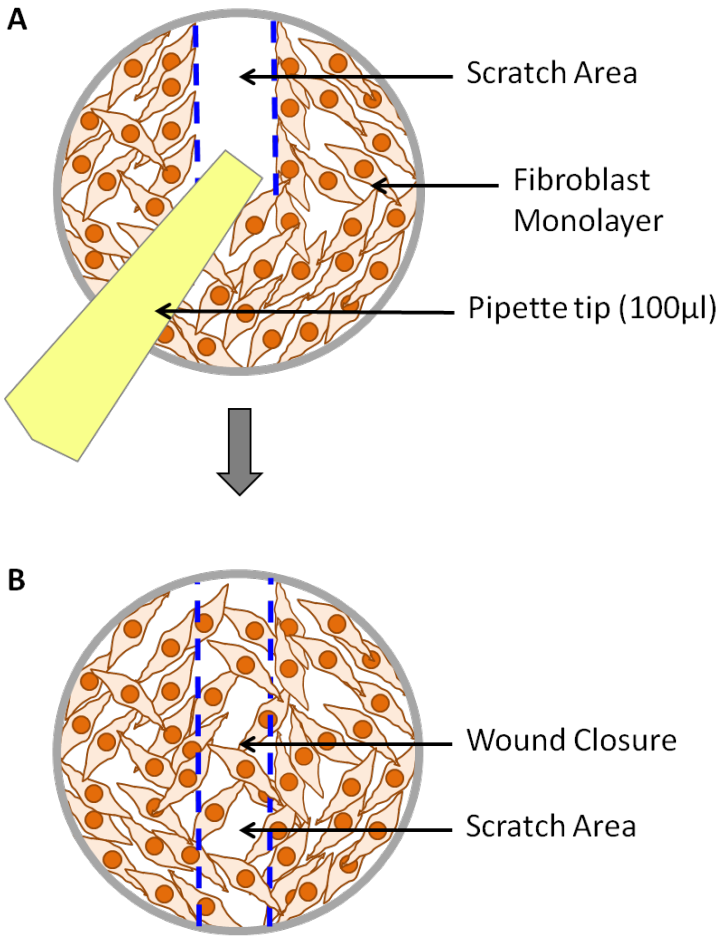


Figure 2.7 Schematic representation of the *in vitro* scratch wound assay.

(**A**) A single scratch in the monolayer of cells was performed using a 100µl pipette tip. The resulting cell-free area, indicated by the blue dotted line, was termed as the 'scratch area'. (**B**) The scratch cell monolayer was imaged at 24-hour intervals to assess the scratch area and the potential movement of cells into the scratch area. The movement of cells into the scratch area was thought to be due to migration and/or proliferation and was termed 'wound closure'.

2.13.1 Analysis of the scratch wound area using ImageJ software

To measure the scratch area, images of a scratch wound that were taken at 0-, 24- and 48-hour intervals were opened, in a consecutive manner, in ImageJ. These images were then stacked into one file (**Image-->Stacks-->Images to Stack**) and then converted to an **"8-bit"** image before saving the file. The saved file with all three images of the scratch wound at 0, 24 and 48 hours was then defined (**Process-->Sharpen** and **Process--> Find Edges**) and the threshold was adjusted to produce a black and white image (**Image-->Adjust-->Threshold**), which allowed for a suitable contrast between cells and the blank scratch area. The image was defined again (**Process--> Find Edges**) before using the **Wand (tracing) tool** to outline the scratch area (shown by a yellow line). The area was then measured (**Analyse-->Measure**), which appeared in a summary window. Measurements of the scratch area were taken of 0-, 24-, and 48-hour images, for all treatment conditions. Wound closure was determined as percentage closure (%), which was subsequently calculated as follows:

$$\left[\frac{(\text{Scratch Area at 0 hours}) - (\text{Closed Scratch Area})}{(\text{Scratch Area at 0 hours})} \right] \times 100$$

2.14 Cell Viability Assay

The cell viability of HEKs and HDFs was quantified using PrestoBlue™ Cell Viability Reagent Protocol (Life Technologies, Paisley, UK). Cells were seeded into the wells of a microtiter plate at a cell density of 1 x 10^4 cells per well and were cultured in DMEM (complete) or EpiLife overnight, or until 95% confluency was reached. Culture

medium was then removed and a scratch was created, as with the scratch wound assay, however with a 10µl pipette tip. The cells were washed with PBS before adding either 100µl of control medium (either DMEM complete or EpiLife) or treating the cells with 100µl of bacterial-conditioned medium per well. To assess cell viability, treatments were removed from the cells before briefly washing the cells in PBS and adding 90µl of control medium (either DMEM complete or EpiLife) to the cells. The PrestoBlue™ reagent was then added to each well (10µl) and incubated for 30 minutes at 37°C. The fluorescence of each well was then read (excitation 525nm) using the GloMax®-Multi+ Microplate Multimode Reader with Instinct® (Promega, Southhampton, UK). The PrestoBlue™ reagent was discarded and the cells were washed with PBS before replacing the control and bacterial-conditioned medium treatments and further incubating. Cell viability was measured at 4-, 24- and 48-hour time points. Each treatment was performed in duplicate. Average fluorescence units were plotted against each experimental setting (E.g. Control, *P. aeruginosa* PCM) at each of the various time points.

2.15 3D Keratinocyte-Fibroblast Co-culture Model

2.15.1 Creating the fibroblast dermal layer

To create the dermal layer of the skin construct, HDFs were grown in a fibrin matrix. Human fibrinogen (depleted of plasminogen, von Willebrand factor and fibronectin) (Enzyme Research Laboratories, Swansea, UK) was diluted in PBS with calcium and magnesium to a concentration of 4 mg/ml. The fibrin gel was created by mixing 2.1ml of the fibrinogen solution with 157µl PBS and 265µl of a 3 x 10^5 cells/ml suspension of HDFs. Bovine thrombin was diluted in a 0.1% BSA solution to a concentration of 5 units/ml before adding 107µl of thrombin to the fibrinogen suspension. The HDF-containing solution was added to a Corning 24mm cell culture insert with a 3µm pore size and polyester membrane (Scientific Laboratory Supplies, East Riding of Yorkshire, UK), where it was left to polymerise. The fibroblast-fibrin gel was submersed in 3ml of DMEM containing 10% FBS, 100 units/ml penicillin-streptomycin (Life Technologies, Paisley, UK), 0.5µg/ml Fungizone®Amphotericin B

(Life Technologies, Paisley, UK) and 10mM HEPES buffer (Life Technologies, Paisley, UK) and incubated in atmospheric, humidified conditions with 5% CO_2 at 37°C. The fibroblasts were incubated for 6-7 days before adding the keratinocytes. The culture medium was changed daily.

2.15.2 Creating the keratinocyte epidermis

The fibroblast dermal layer was washed in PBS without calcium and magnesium three times to remove traces of the DMEM culture medium before adding the HEKs. A keratinocyte cell density of 1 x 10^6 cells was added to the top of the fibroblast dermal layer and then submerged in EpiLife medium (Life Technologies, Paisley, UK) containing HKGS (Life Technologies, Paisley, UK), 100units/ml penicillin-streptomycin (Life Technologies, Paisley, UK), 0.5µg/ml Fungizone®Amphotericin B (Life Technologies, Paisley, UK) and 200 units/ml of the protease inhibitor aprotinin for approximately 2-3 days, or until the HEKs formed a confluent layer. EpiLife medium was changed twice daily.

When the HEKs formed a confluent layer, the skin construct was raised above the air liquid interface, leaving part of the dermal layer submersed in culture medium (see **Figure 2.8**). At this point the culture medium was changed to a 3:1 mixture of DMEM (with phenol red) (Life Technologies, Paisley, UK) and Ham's F12 medium (Life Technologies, Paisley, UK) and was supplemented with 100 units/ml penicillin-streptomycin (Life Technologies, Paisley, UK), 5µg/ml human transferrin, 10ng/ml epidermal growth factor (EGF), 200 units/ml aprotinin, 400ng/ml hydrocortisone, 1.8 x 10^{-4}M adenine, 5µg/ml insulin, 50µg/ml L-ascorbic acid and 2 x 10^{-7}M triiodo-L-thyronine sodium salt. The culture medium was changed twice daily for 10 days before transferring the cell culture insert to a Deep-Well plate (BD Biosciences, Oxford, UK) and adding 3:1 DMEM and Ham's F12 medium without the addition of antibiotics and aprotinin. The constructs were cultured for a further 4 days with daily changes of medium, before being treated with bacterial-conditioned medium and fixed for immunohistochemistry (see **2.17**).

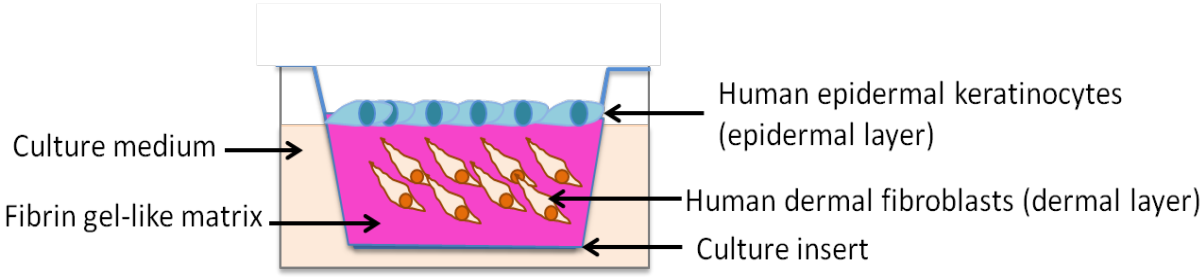


Figure 2.8 Schematic of the 3-dimensional co-culture human skin model.

Human dermal fibroblasts (HDFs) were suspended in a fibrinogen-thrombin solution, before adding the fibroblast-containing solution to the cell culture insert and allowing for the solution to set. The cell culture insert containing the fibroblasts in the fibrin gel matrix was suspended in supplemented DMEM culture medium for 6-7 days. The fibroblast-fibrin gel was termed the 'dermal layer'. The dermal layer was washed before adding human epidermal keratinocytes (HEKs) to the top of the surface. The keratinocytes were cultured in EpiLife medium until confluent before then raising the keratinocytes above the air-liquid interface and continuing to culture for a further 14 days. The keratinocyte layer was termed the 'epidermal layer'. The skin model was then removed from culture and immersed in 10% formalin fixative in preparation for histological analysis.

2.16 Quantitative-RTPCR

2.16.1 RNA Extraction

Cells in monolayer culture were washed in PBS before 1ml of TRIzol reagent (Life Technologies, Paisley, UK) was added to each 6-well plate. The suspension was carefully mixed by pipetting to lyse the cells before transferring to an RNase-free centrifuge tube and storing at -80°C until needed for RNA extraction.

Frozen samples were incubated at room temperature for 15 minutes to allow for the dissociation of the nucleoprotein complex, before adding 200µl of chloroform to each sample. Samples were vortexed and incubated at room temperature for 10 minutes before centrifugation at 12,000 x g for 15 minutes at 4°C. The upper, clear, aqueous phase of the sample was transferred to a clean centrifuge tube, being careful not to take up any of the interphase layer. Isopropanol (500µl) was added to each sample and incubated at room temperature for 10 minutes before centrifugation at 12,000 x g for 10 minutes at 4°C. One ml of 75% molecular grade ethanol made up with 0.1% Diethyl Pyrocarbonate (DEPC) water was added to the samples and gently mixed. The samples were then transferred to a RNeasy Mini spin column and collection tube, part of the RNeasy Mini Kit (Qiagen, Manchester, UK) before centrifugation at 8000 x g for 15 seconds. The flow-through was discarded and 350µl of Buffer RW1 was added to the RNeasy column before centrifugation for 15 seconds at 8000 x *g*. Flow-through was discarded before adding 10µl of DNase I stock solution (Qiagen, Manchester, UK) and 70µl Buffer RDD to the column membrane and incubating at room temperature for 15 mins. Incubation was followed by another Buffer RW1 wash step. Buffer RPE (500µl) was added to the column and centrifuged for 15 seconds at 8000 x g before repeating this step and extending centrifugation to 2 minutes at 8000 x g. The column membrane was dried through centrifugation of the spin column with a new collection tube, at 11,000 x g. RNA was obtained by adding 30µl of RNase-free water to the membrane followed by centrifugation for 1 minute at 8000 x g. RNA concentration was calculated using the Thermo Scientific Nanodrop® ND-1000 spectrophotometer (Fisher Scientific, Loughborough, UK).

2.16.2 cDNA Synthesis

cDNA was prepared by adding 1µg of RNA to 1µl of Random Primers (Promega, Southampton, UK) which was made up to a volume of 13.4µl with 0.1% DEPC water. Samples were heated to 70°C for 5 minutes in a PCR thermo-cycler (Applied Biosystems®, 2720, Life Technologies, Paisley, UK) before adding 5µl of 5X Moloney

Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) Buffer (Promega, Southampton, UK), 1.25µl dATP (10mM) (Promega, Southampton, UK), 1.25µl dCTP (10mM) (Promega, Southampton, UK), 1.25µl dTTP (10mM) (Promega, Southampton, UK), 1.25µl dGTP (10mM) (Promega, Southampton, UK), 0.6µl RNasin® Plus RNase Inhibitor (Promega, Southampton, UK) and 1µl M-MLV RT (Promega, Southampton, UK) to each sample. Samples were heated for 60 minutes at 37°C, followed by 5 minutes at 95°C. cDNA concentration was determined using the Thermo Scientific Nanodrop® ND-1000 (Fisher Scientific, Loughborough, UK).

2.16.3 Quantitative Reverse Transcriptase Polymerase chain Reaction (qRT-PCR)

Primers for human MMP-1, MMP-2, MMP-9, MMP-13 and Collagen-I (see **Table 2.1**) were used to assess regulation at a transcriptional level. The housekeeping gene glyceraldehyde 3-phophate dehydrogenase (GAPDH) was used as a control to normalise quantitative gene expression data. Reaction mix was prepared using 12.5µl GoTaq® qPCR master mix and CXR reference dye (Promega, Southampton, UK), 2.5µl forward and reverse primers and 5µl nuclease-free water per sample. A total of 5µl of cDNA per sample was added to each well of a 96-well MicroAmp® Fast Optical Reaction Plate (Applied Biosystems®, Life Technologies, Paisley, UK) followed by 20µl of the reaction mix. Nuclease-free water (Promega, Southampton, UK) was used as a negative control. All samples were performed in duplicate per assay.

Target (Human)	Forward Sequence (5'-->3')	Reverse Sequence (5-->3')
MMP-1	CTGGCCACAACTGCCAAATG	CTGTCCCTGAACAGCCCAGTACTTA
MMP-2	AGGGCACATCCTATGACAGC	CCTTCTGAGTTCCCACCAAC
MMP-9	CAGAGATGCGTGGAGAGTCGAAA	GGCAAAGGCGTCGTCAATCA
MMP-13	TCCCAGGAATTGGTGATAAAGTAGA	CTGGCATGACGCGAACAATA
Collagen-I	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC

Table 2.1 Primer sequences.

Primers were stored at -20°C and diluted to 3µM in nuclease-free water (Promega, Southampton, UK).

2.17 Histology and Immunohistochemistry

Immunohistochemistry was performed on equine normal skin (n=8), chronic wound-derived granulation tissue (n=8) and the 3D co-culture skin model (see **2.12**), which was performed as part of a service provided by the on-site Veterinary Pathology Department. All tissue samples were fixed in 10% neutral-buffered formalin for a minimum of 24 hours. Fixed samples were processed in a series of ethanol dehydration steps, a clearing xylene step to displace residual ethanol and remove fats, and then finally infiltrated with paraffin wax. Following processing, samples were embedded in paraffin wax blocks and sectioned (4-6 micron sections) onto slides. Routine haematoxylin and eosin stains were performed on all specimens. A Gram stain for normal and chronic equine tissue samples was performed to identify any presence of bacteria within the tissue.

For immunohistochemistry staining, unstained sections were mounted onto slides coated with poly-L-lysine. Sections were deparaffinised in xylene for 5 minutes, three times. The re-hydration of sections was performed by immersing the slides three

times 100% ethanol for 3 minutes, twice in 95% ethanol for 3 minutes, twice in 80% ethanol for 3 minutes and then rinsing slides in distilled water for 5 minutes. Sections were blocked in 10% goat serum in PBS for 1 hour at 25°C in a humidified chamber. Excess blocking solution was removed and 100µl of primary antibody, diluted in 1% goat serum in PBS, (Goat anti-rabbit MMP-2: 1 in 200, Goat anti-rabbit MMP-9: 1 in 200, Goat anti-rabbit MMP-13: 1 in 100) was added to the slide and incubated overnight at 4°C in a humidified chamber. Excess primary antibody was removed and the slides were washed three times in PBS for 5 minutes each wash. Diluted secondary antibody was added to the slides and incubated for 1 hour at 25°C in a humidified chamber. Slides were washed four times in PBS for 5 minutes each wash before adding a colour developer. Slides were washed in PBS an additional four times for 5 minutes each wash before a final wash in distilled water for one minute. Slides were dehydrated in 80% ethanol for one minute, 95% ethanol for one minute and 100% ethanol for one minute. Each ethanol step was repeated in fresh ethanol. Slides were washed in xylene three times for one minute each wash. Coverslips were applied to the slides using mounting medium. Stained sections were visualised using the Nikon 80i Eclipse advanced research microscope.

2.18 Statistical Analysis

Statistical analysis of data from the non-specific protease assays, scratch wound assays, cell viability assays, quantitative RT-PCR data and crystal violet assays was performed using Graphpad Prism® Version 6.0. Specifically, data from the non-specific protease assays and quantitative RT-PCR was statistically analysed using the Kruskal-Wallis non-parametric analysis of variants (ANOVA) with a Dunn's multiple comparison test. Data from the scratch wound assay and cell viability assays were assessed using a two-way ANOVA with a Tukey's multiple comparisons test. The analysis of ammonium sulfate fractions using the non-specific protease assays was statistically assessed using Freidman's non-parametric ANOVA with a Dunn's multiple comparison test.

CHAPTER 3:

Results I: The Proteolytic Activity of Bacteria Isolated from Acute and Chronic Equine Wounds

3 Results I: The Proteolytic Activity of Bacteria Isolated from Acute and Chronic Equine Wounds

3.1 Introduction

The detrimental aspects of bacterial infection in human wounds have long been recognised and their control is crucial for wound management. However it is now thought that the presence of biofilms, complex communities of microorganisms that reside in a self-synthesised matrix, are responsible for the development of chronic outcomes in many wounds in humans (James et al. 2008). In human medicine, biofilms are a great burden on healthcare establishments due to their association with antibiotic resistance. Medical biofilms, as they are referred to, have been detected on indwelling medical devices such as catheters and implant materials, and have been associated with septicaemia, osteomyelitis and periodontitis (Gristina et al. 1985a, Singhai et al. 2012, Distel et al. 2002, Mohamed et al. 2004).

In terms of veterinary medicine, biofilms, particularly in the context of chronic wounds, are greatly under-researched. Indeed, the development of chronic wounds is prevalent in horses. Horse wounds can be categorised as either surgical or traumatic, and then further diagnosis of the healing status of the wound can be described as acute or chronic (Westgate et al. 2011). Traumatic wounds in horses can arise from the horse's immediate environment such as damaged fencing, kick injuries or activities such as cross-country jumping and racing. Surgical wounds, as the name suggests, occur as a result of invasive surgery. Both traumatic and surgical wounds can show signs of delayed healing due to factors such as ischemia, the horse's nutritional state, wound pH and wound location (Westgate et al. 2010). Equine distal limb wounds, are notoriously difficult to heal when compared to wounds on the proximal limb, or main body, which has been described in ponies. This is predominantly due to a greater deal of wound retraction in the limbs than the main trunk of the body (Jacobs et al. 1984).

Due to the nature of the environment where horses reside, they are exposed to many environmental pathogens and therefore there is an obvious risk of infection

and biofilm formation in lower limb wounds, potentially delaying wound closure further. However this is yet to be fully investigated in equine wounds. Cochrane and colleagues identified small clusters or aggregates of bacteria in skin biopsies taken from equine wounds, which demonstrated the presence of biofilms in horse wounds for the first time. This study identified *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* to be the most common isolates from the equine wound (Cochrane et al. 2009). Westgate and co-workers identified Gram-positive and Gram-negative bacterial aggregates in 61.5% (8 of 13) equine wounds, with the predominant species in these wounds being *P. aeruginosa* and *Enterococcus faecium*, and *Staphylococcus* being the most commonly isolated genus (Westgate et al. 2011). A study into the presence of biofilms on the surgical sutures of wounds in cats, dogs and horses, identified Gram-positive biofilms using staining techniques and microscopy. However, of the 91 tissue samples examined, only two contained bacterial colonies resembling a biofilm from canine tissue (König et al. 2014). These results do not support the findings of Westgate and colleagues and also in human studies, whereby it was shown that approximately 60% of chronic wounds contained a biofilm (James et al. 2008). Although König and colleagues assessed samples from tissue that had been categorised as having inflammation, it is still unclear as to whether this presence of inflammation was in an acute or chronic setting. Whilst the identification of equine wound-derived biofilms has been achieved microscopically in these studies, the clinical assessment and diagnosis of biofilms in equine wounds relies on the identification of a thick, shiny and slimy layer on the surface of the wound (Westgate et al. 2010). Although there is evidence to suggest that the biofilm resides in this layer of slime (Cochrane et al. 2009) histological evidence from the research discussed, has shown bacterial biofilms that reside deep within the tissue.

It has been demonstrated that there is an imbalance of matrix metalloproteases (MMPs) and their inhibitors, tissue inhibitors of metalloproteases (TIMPs) in human chronic wounds, and wound fluid has shown to have a significant up-regulation in MMPs (Yager et al. 1996). A similar theory has been proposed in the context of equine chronic wounds (Clutterbuck et al. 2010), although there is a lack of relevant research studies. Nevertheless, if equine chronic wounds also share a similar

dysregulation in the proteolytic environment of the wound, it is important to consider the contribution of bacterial proteases. Indeed, bacterial proteases have been implicated in human pathologies, including chronic lung infections, whereby *P. aeruginosa* elastase B (LasB) has been shown to degrade airway mucins, an important component of the mucus barrier defence against microorganisms (Henke et al. 2011). In equine pathology, the *Bacillus*-derived metalloprotease, thermolysin, has been shown to play a role in the lamellar explant separation and host MMP activation in an *ex vivo* model of equine laminitis (Mungall et al. 2001).

As of yet, there appears to be no evidence of proteolytic activity in bacterial isolates taken from equine chronic wounds. It was therefore the aim of this chapter to assess the proteolytic activity of *P. aeruginosa* and *S. aureus* isolated from acute and chronic wounds of the horse.

3.2 Methods

The bacterial isolates used in this chapter were derived from equine acute and chronic wounds, including limb wounds and surgical wounds (see **Table 3.1**), and were isolated and identified as part of a previous study at the University of Liverpool (Westgate et al. 2011). For a detailed description of the methods used in this chapter, the reader is directed to Chapter 2 of this thesis (2.3 Microbiology, 2.3.6 Crystal Violet Biofilm Forming Assay, 2.5 Scanning Electron microscopy, 2.6 Non-specific Protease Assays, 2.7 Gel Electrophoresis and 2.18 Statistical Analysis).

3.3 Results

3.3.1 Growth curve analysis and biofilm forming potential of equine acute and chronic wound-derived P. aeruginosa and S. aureus

Growth curves of *S. aureus* and *P. aeruginosa* isolates from both acute and chronic wounds of the horse (see **Table 3.1**) were taken to assess their cell proliferation over a period of time. Growth curves within this experiment were performed in a 96-well

plate format, whereby the absorbance of each well was measured at several time points over a 72-hour period. *S. aureus* isolates followed similar growth curve profiles to each other, with most isolates entering the exponential phase between 0 and 16 hours (see **Figure 3.1**, **A**). Beyond this time frame, the *S. aureus* isolates appeared to stay within the stationary phase up to 72 hours. Two out of the seven, 'Mgg' and 'Gj', *S. aureus* isolates showed a higher amount of proliferation than the other isolates. Similarly, the *P. aeruginosa* isolates all followed similar growth curve profiles to each other, with 'Ha14' and 'Ba19' showing a slightly higher amount of proliferation than other isolates (see **Figure 3.1**, **B**). Most of the *P. aeruginosa* isolates entered the exponential phase of growth between 0 and 12 hours, however both 'Ha14' and 'Ba19' reached 20 hours before exponential growth reached a plateau. *P. aeruginosa* isolates entered the stationary phase between 12 and 32 hours respectively, before a decline in the growth curves was seen, indicating cell death. No growth was detected in the DMEM control throughout the duration of the experiment (see **Figure 3.1**, **A** and **B**).

The next experiment tested the ability of each *S. aureus* and *P. aeruginosa* clinical isolate to form a biofilm on plastic pegs within a 96-well plate, as described in Chapter 2 of this thesis. Within this experiment, the equine wound-derived isolates were cultured in the assay for 72 hours before assessing biofilm-formation on the pegs. Crystal violet was used to stain the biofilms before solubilisation, whereby the denser the biofilm, the stronger the intensity of the dye. The majority of *P. aeruginosa* isolates displayed strong biofilm forming potential, shown by the high absorbance values from the crystal violet staining (see **Figure 3.2, A**). Variability was seen between *P. aeruginosa* isolates, with isolates such as 'Ma3' and 'Mt' showing relatively weak biofilm formation. *S. aureus* isolates showed particularly weak biofilm formation with negative absorbance values for the majority of isolates (see **Figure 3.2, B**).

Scanning electron microscopy of *S. aureus* and *P. aeruginosa* grown as biofilms on membrane filter discs also support the crystal violet findings. The spherical cocci *S. aureus* appeared scattered with the occasional presence of small clusters, indicative of biofilm formation (see **Figure 3.3, A and B**). However whilst clustering amongst *S.*

aureus was seen, the surface of the membrane filter was still visible. *P. aeruginosa* however, formed dense layers, with the rod-like morphology of the bacteria barely visible on the surface of the biofilm. *P. aeruginosa* appeared to be surrounded by a extracellular polymeric substances (EPS), thus making it more difficult to see the morphology of the bacteria (see **Figure 3.3, C and D**).

Isolate Code	Type of Wound (Acute/Chronic)	Wound Origin
Staphylococcus aureus		
Gj	Acute	S
Kc	Acute	T
Ke	Chronic	T
Lb	Chronic	T
Mgg	Chronic	T
Tg	Chronic	T
Wi	*Normal Equine Skin*	-
Pseudomonas aeruginosa		
Ba19	Chronic	T
Ca1	Chronic	T
Ha14	Chronic	T
Ia7	Chronic	T
Ma1	Chronic	T
Ma3	Chronic	T
Mt	Acute	S

Table 3.1 Origin of equine bacterial isolates.

P. aeruginosa (n=7) and *S. aureus* (n=7) were isolated and identified from traumatic (T) and surgical (S) equine wounds, which were classified as either acute or chronic. These isolates were obtained from a previous study held at the University of Liverpool by Westgate and colleagues (Westgate et al. 2011).

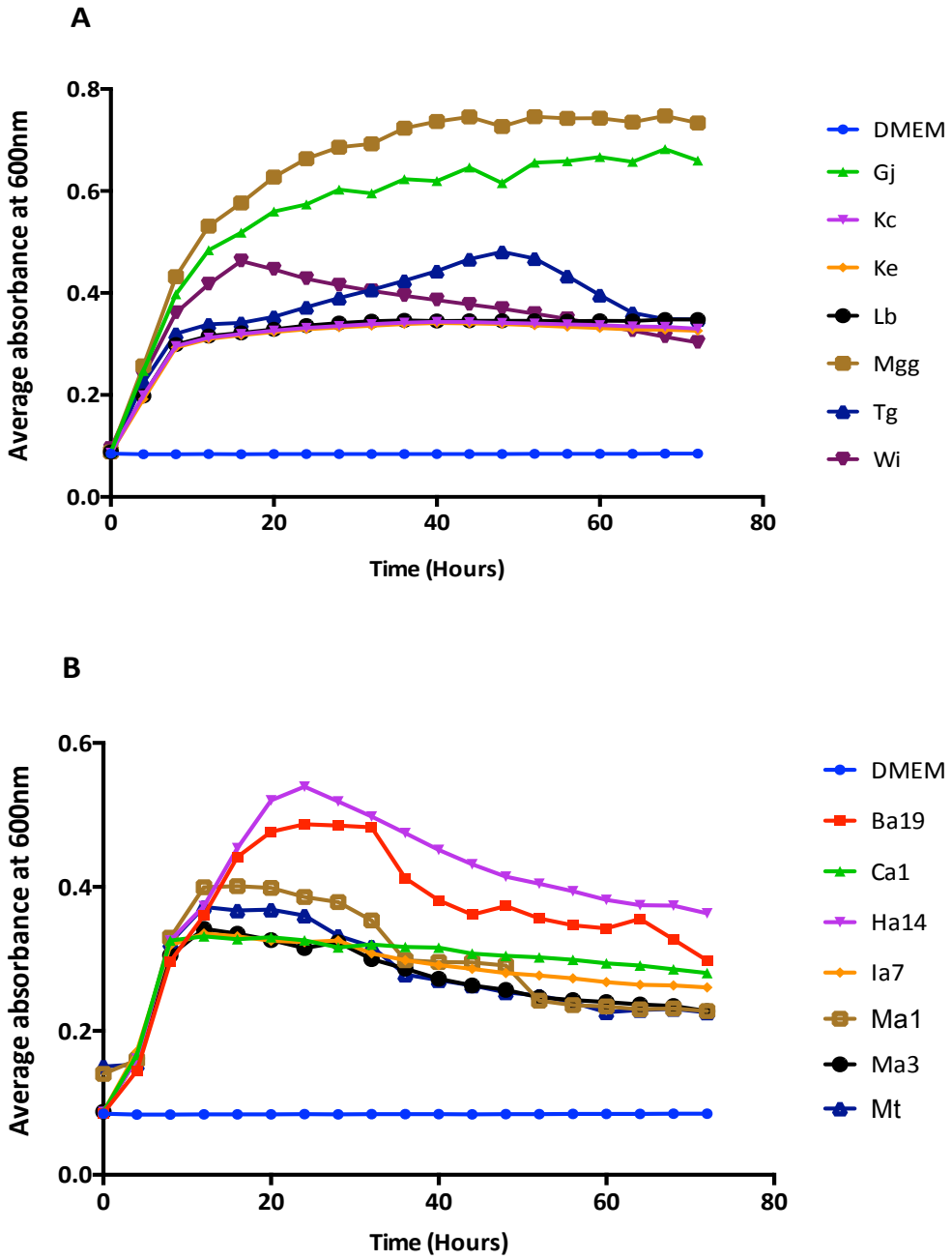


Figure 3.1 Growth curves of equine-derived *S. aureus* and *P. aeruginosa* over 72 hours.

S. aureus (n=7) (**A**) and *P. aeruginosa* (n=7) (**B**) clinical isolates were individually seeded into a 96-well plate and incubated for 72 hours, whereby the absorbance of each well containing the isolate was measured every 4 hours. Dulbecco's Modified Eagle's medium (DMEM) was used as a negative (no growth) control. The assay was repeated three times and the average absorbance values obtained and used to plot the growth curves.

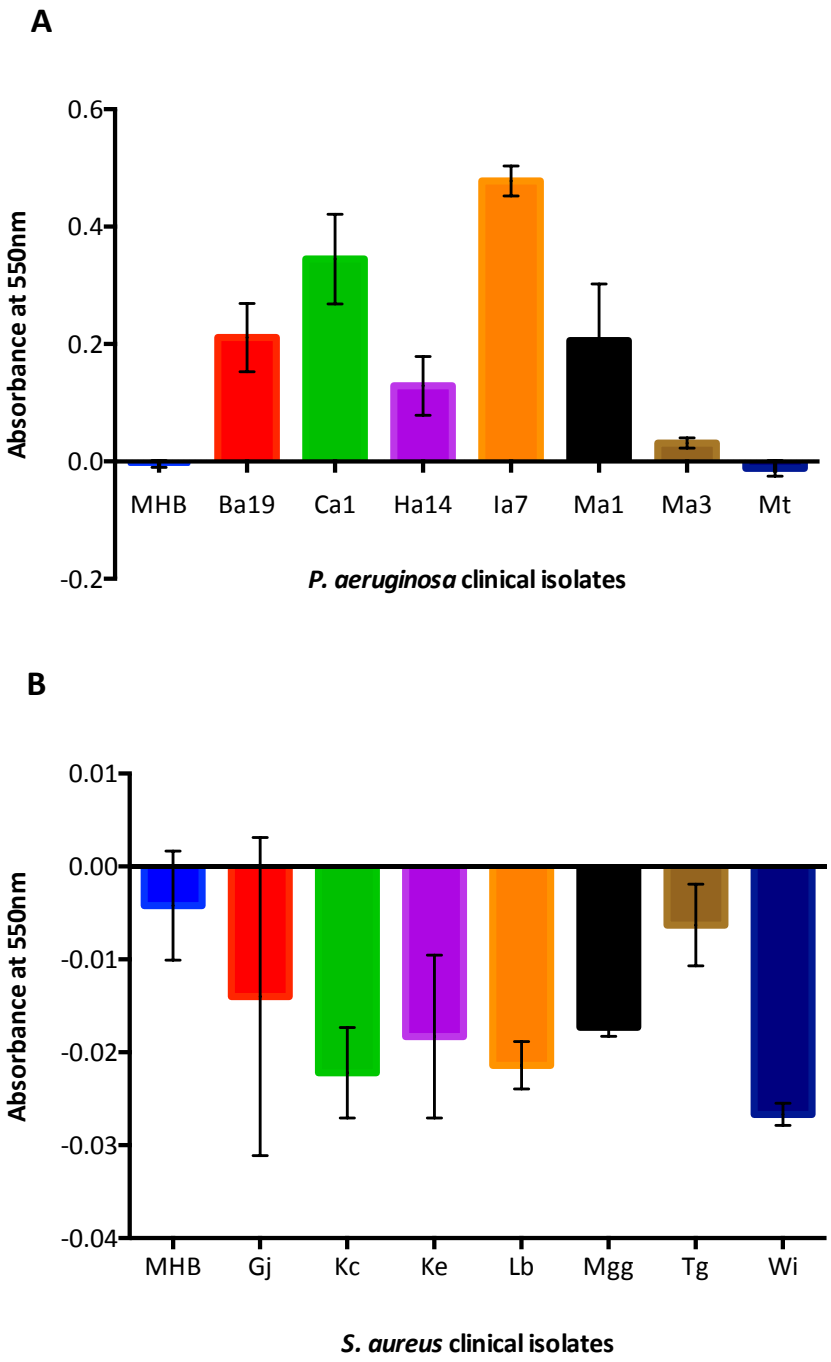


Figure 3.2 Biofilm forming potential of equine-derived clinical isolates using crystal violet.

P. aeruginosa isolates (n=7) (**A**) and *S. aureus* isolates (n=7) (**B**) were incubated for 72 hours before being stained with crystal violet, solubilised and absorbance readings recorded at a wavelength of 550nm. Mueller Hinton broth (MHB) was used as a negative control. The experiment was repeated three times before taking an average of the absorbance values. Error bars represent standard deviation.

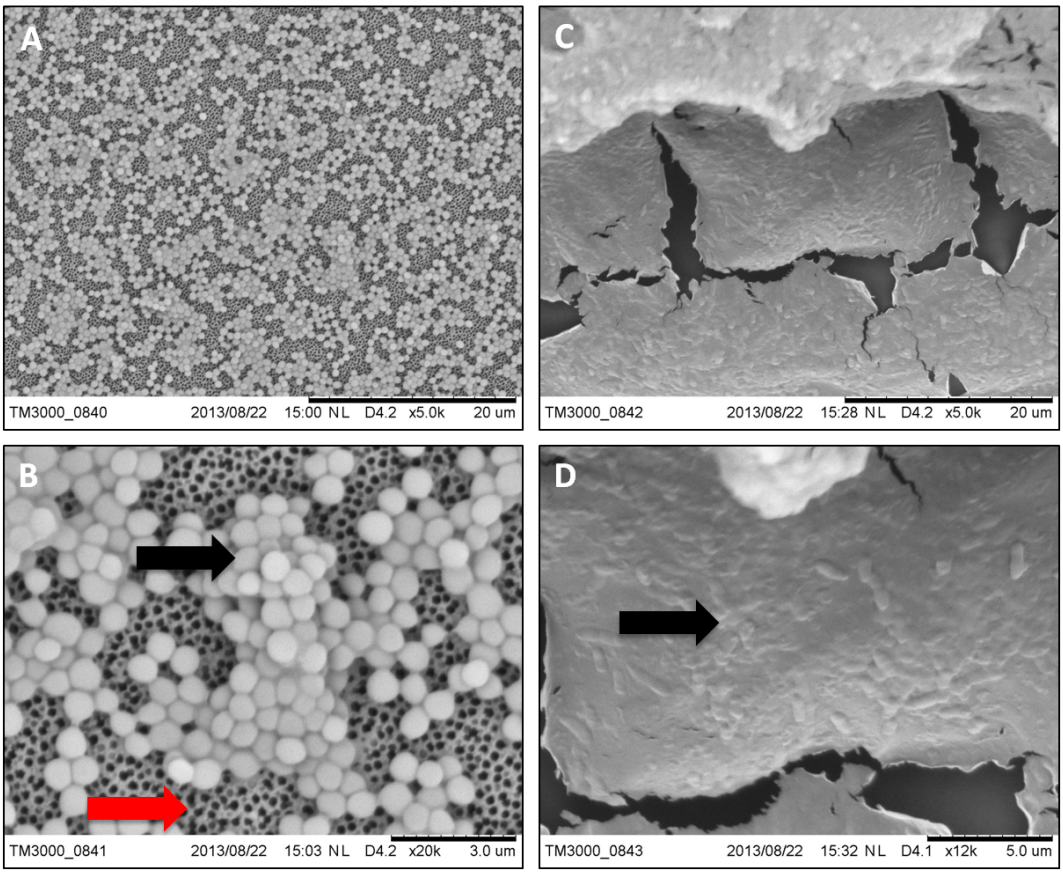


Figure 3.3 Scanning electron microscopy (SEM) of 72-hour biofilms grown on membrane filter discs.

Images represent *S. aureus* 'Tg' at x5000 (**A**) and x20,000 (**B**) magnification and *P. aeruginosa* 'Ba19' at x5000 (**C**) and x12,000 (**D**) magnification. Both isolates were grown on membrane filters for 72 hours before being washed in PBS and fixed in 2.5% gluteraldehyde and dehydrated in ethanol. Images were taken using the Hitachi TM300 tabletop scanning electron microscope. Black arrows indicate evidence of bacterial clustering and the red arrow shows the membrane filter surface.

3.3.2 Proteolytic activity of equine acute and chronic wound-derived P. aeruginosa and S. aureus in planktonic and biofilm form

To assess the general proteolytic activity of the equine-derived *S. aureus* and *P. aeruginosa* planktonic- and biofilm-conditioned medium (PCM and BCM), the substrates Azo-casein and Azocoll were used. The Azo-casein assay revealed low levels of proteolytic activity in *S. aureus* PCM and BCM (see **Figure 3.4**). No differences in proteolytic activity were recorded between *S. aureus* PCM and BCM. On the other hand, both *P. aeruginosa* PCM and BCM showed high levels of proteolytic activity, with *P. aeruginosa* PCM and BCM showing significantly higher activity than *S. aureus* PCM and BCM, respectively ($P < 0.01$ and $P < 0.05$). No differences in proteolytic activity were recorded between *P. aeruginosa* PCM and BCM. Similarly the Azocoll assay revealed low levels of activity in *S. aureus* PCM and BCM, with no significant difference between PCM and BCM (see **Figure 3.5**). *P. aeruginosa* PCM and BCM showed high levels of protease activity, with *P. aeruginosa* PCM showing higher activity than *S. aureus* PCM ($P < 0.01$). Although proteolytic activity appeared higher in *P. aeruginosa* BCM than *S. aureus* BCM, no statistically significant differences were determined.

In order to determine whether the high proteolytic activity seen in *P. aeruginosa* PCM and BCM, was in part, due to the presence of metalloproteases, all bacterial-conditioned medium was run on gelatin zymograms. Zymography revealed the presence of a 52kDa protease, to varying degrees of intensity in most *P. aeruginosa* PCM samples (see **Figure 3.6, A**). This 52kDa protease did not appear in the PCM of the isolate 'Mt'. Furthermore, there were no differences noted in the intensity of the proteolytic bands between *P. aeruginosa* PCM and BCM (see **Figure 3.6, B**), although this was not semi-quantifiably measured. The presence of proteolytic bands was not detected in *S. aureus* PCM and BCM (data not shown). In order to determine which of the major groups of proteases this 52kDa protease belonged to, *P. aeruginosa* PCM was run on several gelatin zymograms and either incubated with a serine protease inhibitor (Benzamidine hydrochloride hydrate), a metalloprotease inhibitor (EDTA) or a cysteine protease inhibitor (N-ethylmaleimide or NEM). The use of 100mM EDTA inhibited the equine fibroblast-derived MMP-2 positive control but

failed to inhibit the *P. aeruginosa*-derived protease bands. Furthermore, 50mM Benzamidine hydrochloride hydrate and 10mM NEM did not have an inhibitory effect on the 52kDa bands when compared to the bands incubated in normal refolding buffer (see **Figure 3.6, A**).

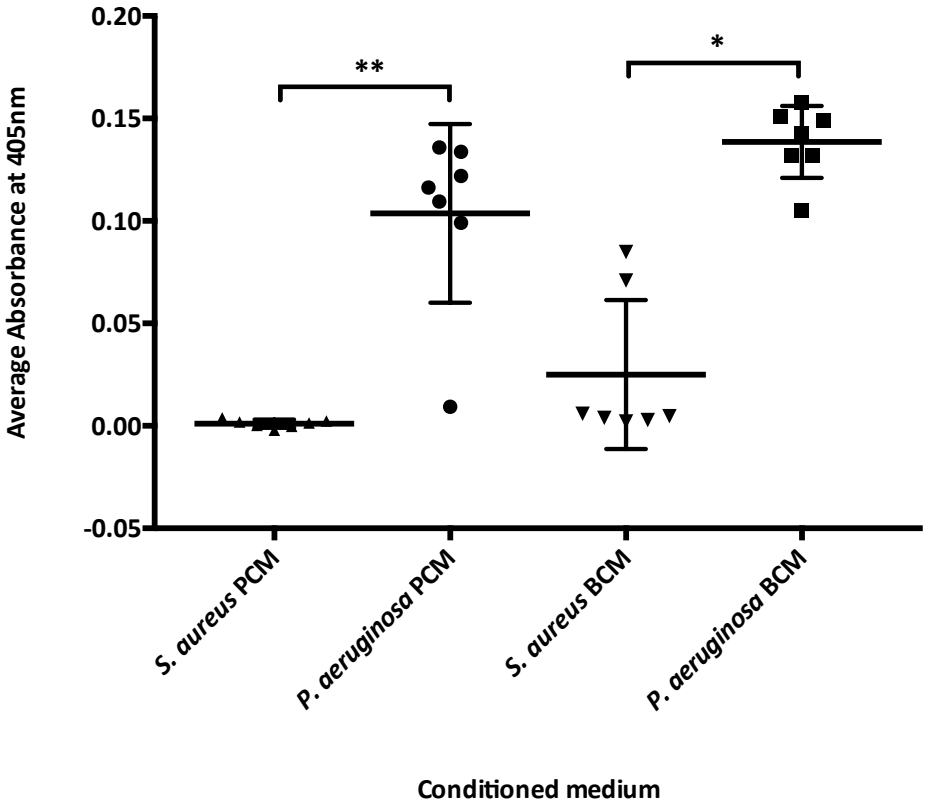


Figure 3.4 The non-specific proteolytic activity of planktonic and biofilm *S. aureus*- and *P. aeruginosa*-conditioned medium using the Azo-casein assay.

Data from the biological replicates were combined in order to assess the statistical difference between clinical *S. aureus (n=7)* and *P. aeruginosa (n=7)*. All biological replicates in the assay were run in duplicate. Error bars represent standard deviation. Statistical analysis was performed using the Kruskal-Wallis non-parametric ANOVA, with Dunn's multiple comparison test; *P < 0.05, **P < 0.01. Abbreviations include planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

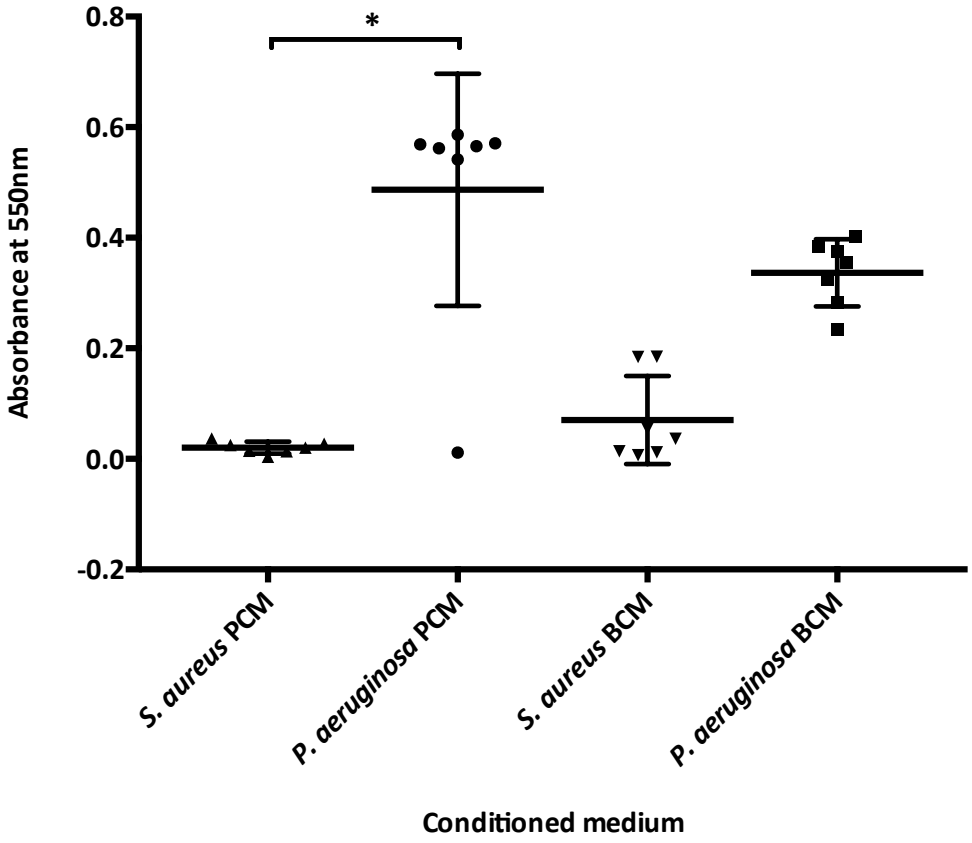


Figure 3.5 The non-specific proteolytic activity of planktonic and biofilm *S. aureus*- and *P. aeruginosa*-conditioned medium using the Azocoll assay.

Data from the biological replicates were combined in order to assess the statistical difference between clinical *S. aureus (n=7)* and *P. aeruginosa (n=7)*. All biological replicates in the assay were run in duplicate. Error bars represent standard deviation. Statistical analysis was performed using the Kruskal-Wallis non-parametric ANOVA, with Dunn's multiple comparison test; *P < 0.01. Abbreviations include planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

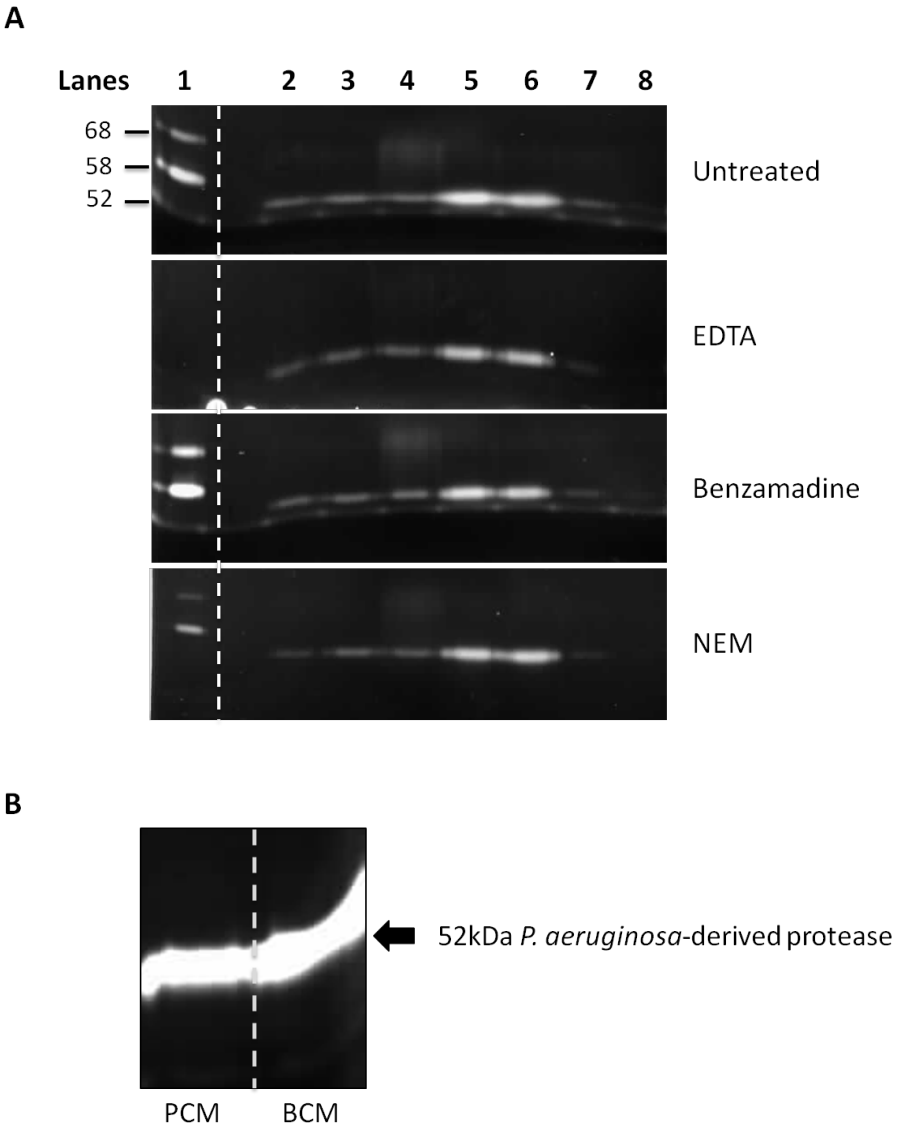


Figure 3.6 The detection and inhibition of equine wound-derived *P. aeruginosa* proteases.

P. aeruginosa PCM was run on 0.25% gelatin and 7.5% poly-acrylamide zymograms (**A**). Lanes 2-8 represents the PCM of equine isolates BA19, Ca1, Ha14, Ia7, Ma1, Ma3 and Mt respectively. Lane 1 shows the MMP-2 positive control, isolated from equine fibroblasts. Zymograms were incubated with either untreated buffer, 100mM EDTA-treated buffer, 50mM benzamidine-treated buffer or 10mM NEM-treated buffer. *P. aeruginosa* PCM and BCM were run on a gelatin zymogram for comparison in the abundance of the protease (**B**). Molecular weight is displayed in kilodaltons (kDa). Protease activity is shown as a white band against a Coomassie blue-stained background. Experiments were performed using all biological replicates of *P. aeruginosa* (n= 7) and were repeated three times. Abbreviations include Ethylenediaminetetraacetic acid (EDTA), N-ethlymaleamide (NEM), planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

3.3.3 Identification of equine-derived P. aeruginosa protease using mass spectrometry

In order to identify the *P. aeruginosa*-derived protease of approximately 52kDa, three biological replicates of *P. aeruginosa* PCM (Ba19, Ca1 and Ma1) were run on a gelatin zymogram. The zymogram was stained to visualise the protease band before the bands were cut out of the gel and used for mass spectrometric analysis. For the control, Mueller Hinton broth (MHB) was run on the same zymogram and a sample of the equivalent molecular weight as the *P. aeruginosa* 52kDa protease was cut out and used for mass spectrometric analysis (carried out by the University of Manchester). Data in the form of amino acid sequences in the protease sample were entered into the Mascot search engine against the SwissProt database under the 'Eubacteria' taxonomy in order to identify potential bacterial-derived proteases. In this experiment, I focused on proteins identified by the Mascot search engine that shared significant identity or shared homology ($P < 0.05$) to the peptide sequence in the protease sample. Therefore, although each sample received protein 'hits', only the results that were deemed significant by Mascot were assessed. Results from the Mascot search showed that all biological replicates of the protease received at least one protein hit, however the results for each protease sample, differed from each other (see **Table 3.2**). The sample 'Ca1' had four protein hits, however these proteins were also detected in the control sample. Protease samples 'Ba19' and 'Ma1' each had one protein hit that were not detected in the control sample. The 85kDa protein DNA translocase ftsK was detected in 'Ma1' and was shown to be associated with the bacterium *Xanthomonas axonopodis pv*. For 'Ba19', a 35kDa protein named S-adenosyl-L-methionine-dependent methyltransferase mraW, associated with the bacterium *Alkaliphilus oremlandii* was detected. Unfortunately, the protein results for all three protease samples showed varying molecular weights dissimilar from the initial 52kDa *P. aeruginosa*-derived protease. Furthermore, there was no evidence of *P. aeruginosa*-derived proteins/proteases.

Protein description	Associated bacteria	Protein Mass (daltons)	Peptide sequence identified	Presence in sample	Presence in control sample
DNA translocase ftsK	*Xanthomonas axonopodis pv*	84,992	KTAAADNPRR	Ma1	No
Protein nrdI	*Klebsiella pneumonia*	15,387	LGLPAVR	Ca1	yes
Exodeoxyribonuclease V beta chain	*Mycobacterium tuberculosis*	119,106	LGLPGLR	Ca1	yes
Lipid-A-disaccharide synthase	*Ralstonia solanacearum*	42,579	LGLPLGR	Ca1	yes
DNA polymerase I	*Bacillus subtilis*	99,030	IGLPVVK	Ca1	yes
S-adenosyl-L-methionine-dependent methyltransferase mraW	*Alkaliphilus oremlandii*	35,303	RAIPKGAR	Ba19	No

Table 3.2 Mass spectrometric identification of the proteins in equine wound-derived *P. aeruginosa* protease samples.

The table shows a list of proteins that have been found to significantly share identity or extensive homology with the peptides within three biological replicates (n=3) of the previously identified *P. aeruginosa* protease sample ($P < 0.05$). For the control, MHB was run on a gelatin zymogram and a sample of the same molecular weight as the *P. aeruginosa*-derived protease was cut out of the gel for mass spectrometric analysis. Peptide sequence data was entered into the Mascot search engine to identify proteins from the SwissProt database under Eubacteria taxonomy. Bacteria associated with the identified protein, according to the Mascot search is listed in the table. Peptide sequences are represented as one-letter symbol amino acids, whereby K= Lysine, T= Threonine, A= Alanine, D= Aspartic acid, N= Asparagine, P= Proline, R= Arginine, L= Leucine, G= Glycine, V= Valine and I= Isoleucine.

3.4 Discussion

The involvement of bacterial proteases in equine wounds has not previously been investigated. For the first time, this study identifies the presence of proteolytic activity from bacteria isolated from both acute and chronic equine wounds using a combination of basic enzymatic assays and substrate zymography. More specifically, *P. aeruginosa* isolates were shown to secrete a 52kDa protease. Unfortunately at this stage of the investigations, there is no definitive answer as to the identification of this protease. Based on the approximate molecular weight of this *P. aeruginosa*-derived protease, the literature suggests that the 52kDa band is that of *P. aeruginosa* alkaline protease (AprA). As discussed in chapter 1 of this thesis, *P. aeruginosa* AprA is a metalloprotease and a known virulence factor secreted by *P. aeruginosa*. A study by Caballero and colleagues, whereby six various *in vitro* assays were used to assess the major *P. aeruginosa* proteases, demonstrated *P. aeruginosa* AprA at a molecular weight of 56kDa in gelatin and casein zymography (Caballero et al. 2001). Another study compared the protease profiles of the *P. aeruginosa* reference strain ATCC 27853 and two clinical strains of *P. aeruginosa*, which displayed different protease profiles in zymography depending on the strain and growth medium used. More specifically, AprA was only detected using gelatin zymography in the strains grown in minimal M9 culture medium but not Luria-Bertani (LB) broth, with a molecular weight of 52kDa (Andrejko et al. 2013). Nevertheless, many studies have reported the inhibition of metalloproteases and indeed, AprA, with the chelator EDTA, which deactivates metalloproteases by sequestering zinc ions (Caballero et al. 2001, Twining et al. 1993). However within this chapter, 100mM EDTA, failed to inhibit the 52kDa *P. aeruginosa*-derived protease, despite successfully inhibiting the MMP-2 positive control. The reason for this remains unknown; however there is a possibility that the 52kDa protease secreted from equine-derived *P. aeruginosa* may display EDTA resistance. An example of EDTA resistance in bacterial metalloproteases has been recorded in *Xenorhabdus*, whereby it was hypothesised that the zinc ion within the metalloproteases may be tightly bound, making the protease inaccessible to EDTA inhibition (Massaoud et al. 2011). Mass spectrometric analysis of the *P.*

aeruginosa-derived protease was based on a sample that was extracted from a gelatin zymogram. While zymography works in the same manner as an SDS-PAGE, whereby the proteins of a particular sample are separated through the gel according to size under the influence of a current, a zymogram contains a substrate, and therefore any proteases that have been separated, effectively digest the substrate in the surrounding gel. Therefore any bands visualised upon staining the gel are amplified, unlike the neat bands that are visualised in an SDS-PAGE gel, resulting in a less accurate isolation of the protease when excising bands for mass spectrometry.

The *S. aureus* isolates in this study showed little proteolytic activity in planktonic and biofilm form. Indeed, *S. aureus* do produce extracellular proteases that play a role in bacterial virulence, such as the metalloprotease aureolysin, the serine protease SspA and the cysteine protease SspB, as mentioned in Chapter 1 of this thesis. Other studies have identified these particular *S. aureus*-derived extracellular proteases using gelatin zymography (Shaw et al. 2004), however the detection of these proteases may be strain-dependant.

There are limitations to the work presented in this chapter. It is important to note that the method used to obtain BCM is based upon a laboratory model of a biofilm, and indeed, like many *in vitro* models, results obtained are only indications of what may occur *in vivo*. For instance, the thickness of the biofilm observed in this model is unlikely to be observed in an equine chronic wound, due to the external environment and host immune responses towards the biofilm. In addition to this, the levels of proteolytic activity from *P. aeruginosa* may differ when these bacteria are within the wound. This could be potentially due to the influence of external factors such as nutrient availability, host-derived factors or surrounding microorganisms.

3.4.1 Future directions

The incorporation of these biofilms into an *in vitro* equine wound healing model will also reveal the effect of the soluble factors released by biofilms on wound closure. More specifically, it would be interesting to investigate how both the PCM and BCM from this chapter affects fibroblast migration and proliferation, given the excessive

production of granulation tissue seen in many chronic equine wounds. Following this, one would be able to determine how host proteases are regulated in response to these external stimuli, in order to create a more complete picture of how equine tissue responds to bacterial infection within a wound. To then assess whether the *P. aeruginosa*-derived exogenous protease highlighted in this chapter contributes to the development of chronic equine wounds, future work would aim to isolate and identify this protease. In doing so, it would determine how this protease is regulated within a biofilm subject to an environment similar to that of the wound site. Further work would then focus on whether this protease impedes wound closure.

3.4.2 Key points

In summary, *P. aeruginosa* isolated from acute and chronic equine wounds displayed high levels of proteolytic activity, which was identified through the use of non-specific protease activity assays. *P. aeruginosa* in planktonic and biofilm form was shown to release a 52kDa protease, detected in gelatin zymography, which was not inhibited by broad spectrum protease inhibitors. The subsequent attempt to identify this protease through the method of mass spectrometric analysis resulted in ambiguous results. Thus, the identification of this protease remains unknown.

S. aureus isolates in both planktonic and biofilm form showed little protease activity and there was an absence of detectable proteases using zymography.

CHAPTER 4:

Results II: The effect of equine wound-derived bacteria on the wound closure and protease production of normal and chronic wound fibroblasts

4 Results II: The effect of equine wound-derived bacteria on the wound closure and protease production of normal and chronic wound-derived fibroblasts

4.1 Introduction

In the aim of understanding how biofilms prevent wound closure, many *in vivo* models of biofilm-infected chronic wounds have been developed. For example, a model using wounded New Zealand rabbit ears has shown sustained low grade inflammation, decreased granulation tissue formation and reduced re-epithelialisation in the presence of *S. aureus* biofilms (Gurjala et al. 2011). Though not all biofilm-infected wound models have resulted in impaired wound healing. Kanno and colleagues infected full thickness wounds with *P. aeruginosa* biofilm in a murine model. The authors visualised biofilm presence on the wounds at as early as 8 hours however there were no differences in epithelialisation when compared to uninfected controls (Kanno et al. 2010). However, it has been proposed that equine models could be a more physiologically relevant model (Theoret and Wilmink 2013). There is a natural occurrence of chronic wounds within the horse due to the nature of the environment that the horse resides, but also, wounds to the limb are often deemed difficult to heal due to the high tension (tightness of the skin) and increased risk of infection (Jacobs et al. 1984). Furthermore, unlike wound healing in the mouse, which predominantly relies on wound contraction rather than epithelialisation, wound healing in the horse is driven more by epithelialisation, drawing parallels to human wound repair. The clinical characteristics of equine chronic wounds shares similarities to those in human, with frequent signs of persistent inflammation and exudate. However a distinct characteristic of chronic wounds on horse limbs is the exuberant production of granulation tissue that requires regular debridement. In human hard-to-heal chronic wounds, there is a suboptimal production of granulation tissue in many cases, potentially due to the high proteolytic environment (Nunan et al. 2014). However similarities can be drawn to human fibroproliferative disorders such as keloids, where excessive scar tissue formation is present. For example, Theoret and colleagues using histological techniques to compare equine granulation tissue and human keloid tissue, found

similarities in dermal thickened collagen fibres and increased numbers of fibroblasts, however there were also differences identified in inflammatory cell types between these tissues (Theoret et al. 2013). Furthermore, significant differences in fibroblasts isolated from normal healthy equine tissue and equine chronic wounds have been noted. Cochrane and colleagues identified morphological differences between normal fibroblasts (NFs) and chronic granulation tissue fibroblasts (GTFs), whereby NFs appear spindle-like and GTFs were spread-out and cuboidal in appearance (Cochrane et al. 1996). Furthermore, the proliferation rates of GTFs were determined significantly lower than NFs after 20 day in culture ($P < 0.01$).

Host proteases have been extensively studied in terms of their role in the mammalian wound healing process. Although it is known that in human chronic wounds, there is an imbalance in matrix metalloproteases (MMPs) and their inhibitors, tissue inhibitors of metalloproteases (TIMPs), there is little research into the expression and regulation of MMPs in horse wounds. When referring to equine chronic wounds, many reviews simply draw parallels to the exaggerated proteolytic environment of human chronic wounds (Clutterbuck et al. 2010), although this may not be the case.

As mentioned previously, bacterial biofilms have been identified in equine wound tissue (Westgate et al. 2011, Cochrane et al. 2009). It has been determined, for the first time, in Chapter 3 of this thesis, that *P. aeruginosa* isolated from equine chronic wounds display high levels of proteolytic activity. However, though the presence of a biofilm within an open wound is understandable, particularly considering the surrounding environment of a horse, it remains to be answered whether these biofilms directly affect wound closure. Furthermore, it remains unknown whether the presence of bacterial biofilms within the wound, affects the secretion of host-derived proteases. Indeed, it has been documented that bacteria and the purified bacterial protease thermolysin, caused the activation of host MMP-2 and MMP-9, leading to lamellar explant separation in an equine laminitis model (Mungall et al. 2001).

The aim of this study was to assess *in vitro* wound closure of equine fibroblasts from normal skin and chronic wound granulation tissue, in response to treatment with *S. aureus* and *P. aeruginosa* planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM), and assess the secretion of host proteases under these experimental conditions. Furthermore, the overall expression of MMPs in equine skin and chronic wound-derived granulation tissue was investigated using immunohistochemistry to determine whether the presence of biofilms in equine chronic wounds affected the overall expression of MMPs.

4.2 Methods

For a detailed description of the methods used in this chapter, the reader is directed to Chapter 2 of this thesis (2.3 Microbiology, 2.7 Gel Electrophoresis, 2.10 Cell Culture, 2.13 Scratch Wound Assay, 2.17 Histology and Immunohistochemistry and 2.18 Statistical Analysis).

4.3 Results

4.3.1 Scratch closure of equine normal and granulation tissue fibroblasts (NFs and GTFs) in response to P. aeruginosa and S. aureus planktonic- and biofilm-conditioned medium

Upon culturing fibroblasts from normal equine skin and chronic wound-derived granulation tissue, differences in cell morphology were identified. Normal fibroblasts (NFs) appeared elongated and spindle-like in shape, whereas granulation tissue fibroblasts (GTFs) appeared cuboidal in shape and in some cases, particularly after treatment with bacterial-conditioned medium, appeared stellate (see **Figure 4.1**, **A** and **B**).

NF's were cultured and seeded into a 6-well plate format to perform an *in vitro* scratch assay. Cultures were either cultured normally in DMEM (complete)

(untreated control) or treated in a 1:1 dilution of either *S. aureu*s or *P. aeruginosa* PCM and BCM. The untreated scratch controls showed complete closure at 72 hours (see **Figure 4.2**, **A**). There was no significant reduction in percentage wound closure in NFs treated with *S. aureus* PCM (49.1% ± 24.8%) and BCM (67.5% ± 15.6%) after 72 hours when compared to the untreated scratch control (97.4% ± 3.6%) (see **Figure 4.3**). After 48 hours, NFs treated with *P. aeruginosa* PCM and BCM showed a significant reduction in percentage closure (-48.3% ± 40.4% and -17.8% ± 63.0%) when compared to the control (78.5% ± 11.4%) ($P < 0.05$ and $P < 0.0001$ respectively) with BCM causing a significant reduction in closure when compared with PCM ($P < 0.0001$). Furthermore a significant reduction in closure was detected in *P. aeruginosa* PCM treated cultures in comparison with *S. aureus* PCM (36.5% ± 14.4%) treated cultures at 48 hours ($P < 0.0001$). After 72 hours, significant differences in percentage closure were still seen in NFs treated with *P. aeruginosa* PCM and BCM (-64.7% ± 29.2% and -17.7% ± 63.0%) when compared with the untreated scratch control ($P < 0.0001$). Furthermore differences in closure were noted between *S. aureus* and *P. aeruginosa* PCM ($P < 0.0001$), *S. aureus* and *P. aeruginosa* BCM ($P < 0.0001$) and *P. aeruginosa* PCM and BCM ($P < 0.01$).

The assessment of scratch closure in fibroblasts isolated from chronic wound granulation tissue, also referred to in this chapter as 'granulation tissue fibroblasts' or 'GTFs', showed complete scratch wound closure after 48 hours, unlike NFs, which took 72 hours to reach complete closure (see **Figure 4.2**, **B**). Control GTF scratches in DMEM (complete) showed an average percentage closure of 77.2% ± 7.9% after 24 hours and 99.5% ± 0.9% closure after 48 hours (see **Figure 4.4**). After 24 hours in culture, cells treated with *S. aureus* PCM showed 55.6% ± 11.1% closure that was significantly reduced when compared to the untreated control ($P < 0.05$). *S. aureus* BCM-treated cells showed a 89.0% ± 7.8% closure after 24 hours, which was significantly higher than percentage wound closure in *S. aureus* PCM- treated cells ($P < 0.0001$). Furthermore, GTFs treated with *P. aeruginosa* PCM and BCM showed significant reduction in scratch closure (12.8% ± 8.1% and 37.6% ± 10.3% respectively) when compared with the control ($P < 0.0001$) and when compared to each other ($P < 0.001$). At 48 hours, cells treated with *S. aureus* PCM and BCM

reached 93.6% ± 9.2% and 99.9% ± 0.1% closure, comparable to the control. Scratch closure of *P. aeruginosa* PCM- and BCM-treated cells was greatly reduced when compared to the control with an average percentage closure of 13.2% ± 10.5% and 16.3% ± 21.2% (P <0.0001).

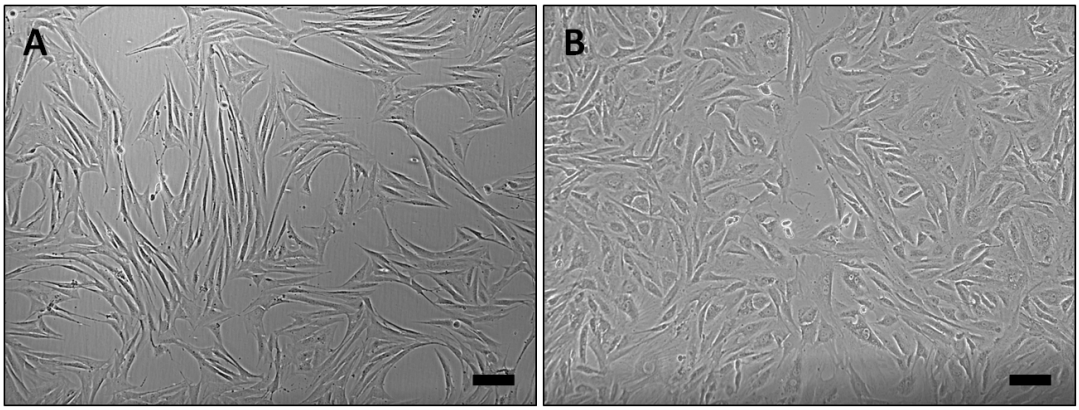
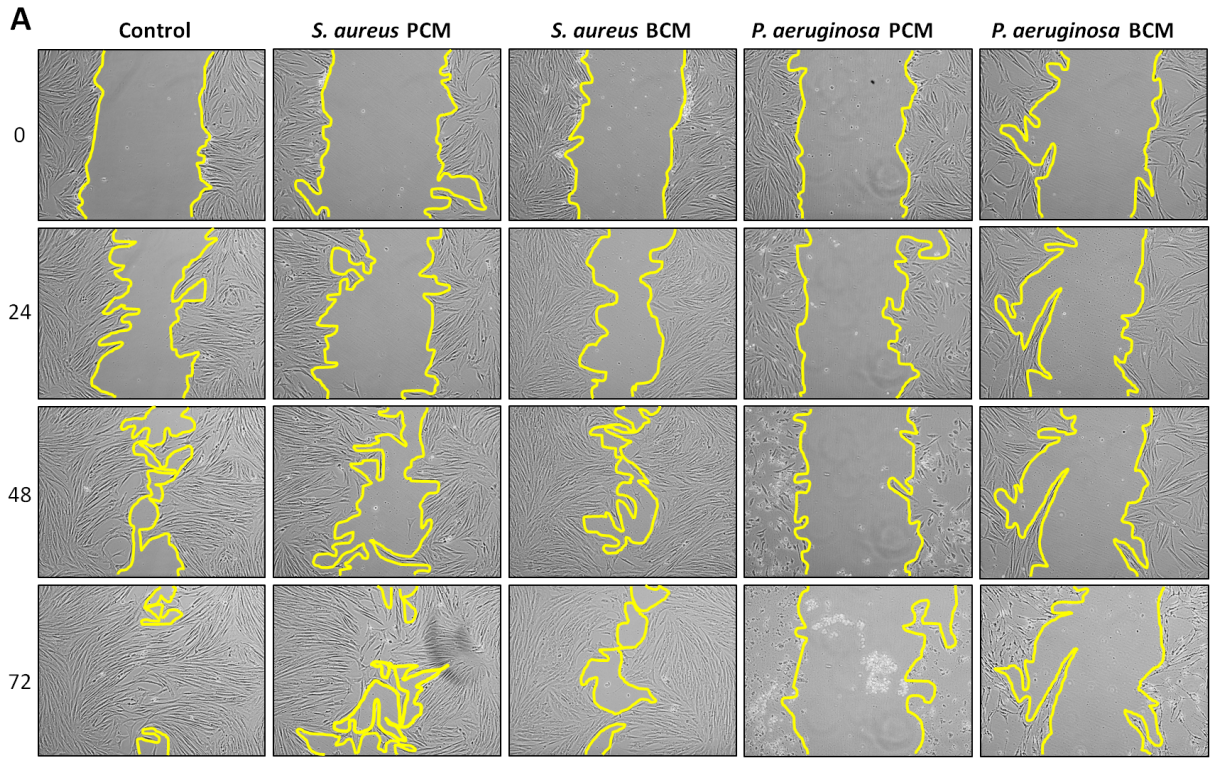


Figure 4.1 Representative images of equine normal skin fibroblasts and chronic wound granulation tissue fibroblasts.

Normal fibroblasts (NFs) (**A**) were isolated from the intact skin of equine lower legs obtained from the abattoir. Granulation tissue fibroblasts (GTFs) were isolated from the granulation tissue of equine chronic lower limb wounds following debridement for clinical purposes. Skin and tissue samples were gently cleaned and cut into 2mm x 2mm pieces before culturing the tissue in supplemented cell culture medium. Fibroblasts could be visualised after 5 days in culture. Images were taken at passage 2 of cell culture at x10 magnification. Scale bar represents 100µm.


A
Control
S. aureus PCM
S. aureus BCM
P. aeruginosa PCM
P. aeruginosa BCM
0
24
48
72

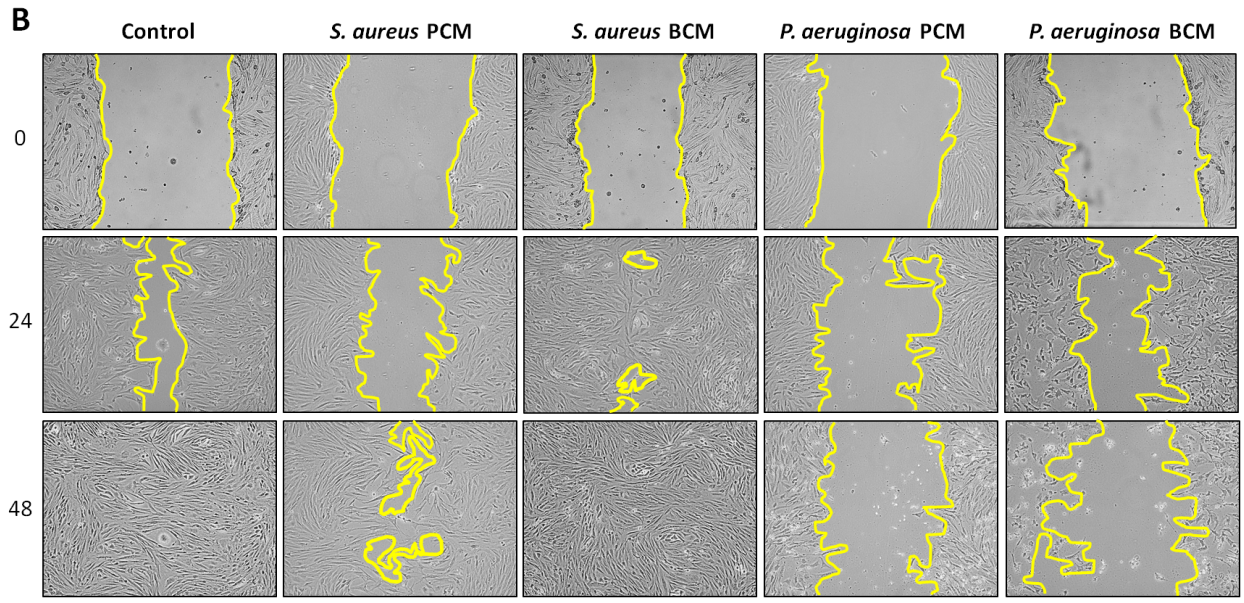


Figure 4.2 Representative images of (A) normal fibroblast (NF) and (B) granulation tissue fibroblast (GTF) scratch wounds after treatment with equine wound-derived *S. aureus* and *P. aeruginosa* planktonic- and biofilm-conditioned medium.

Abbreviations include planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

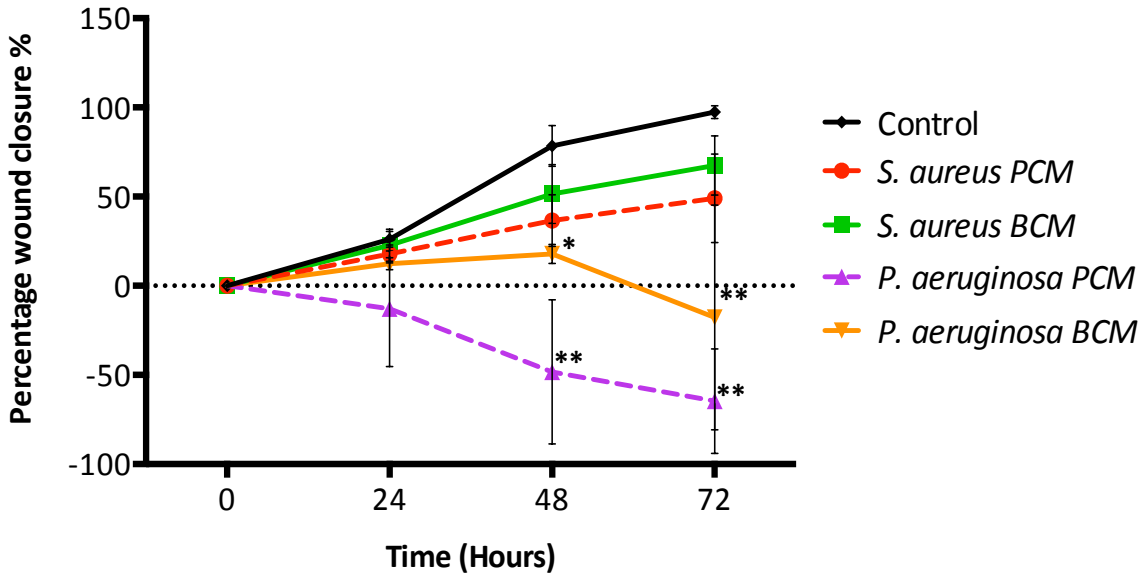


Figure 4.3 Percentage scratch wound closure of normal fibroblasts (NFs) *in vitro*.

Results are shown for NFs treated with either *S. aureus* or *P. aeruginosa* planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM). Results represent the mean values ± standard deviation (biological replicates: *S. aureus* n=7 and *P. aeruginosa* n=7, technical replicates n=3). NFs were used between passage 3 and 5. Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.05 and **P <0.0001 when compared with the control group.

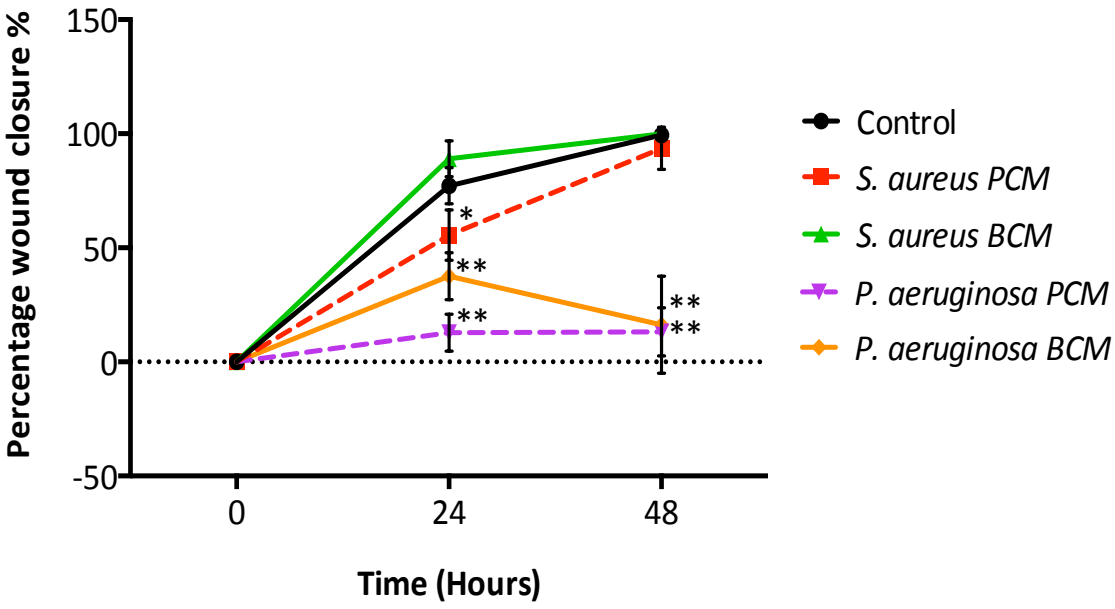


Figure 4.4 Percentage scratch wound closure of granulation tissue fibroblasts (GTFs) *in vitro*.

Results are shown for GTFs treated with either *S. aureus* or *P. aeruginosa* planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM). Results represent the mean values ± standard deviation (biological replicates: *S. aureus* n=7 and *P. aeruginosa* n=7, technical replicates n=3). NFs were used between passage 3 and 5. Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.05 and **P <0.0001 when compared with the control group.

4.3.2 Secretion of equine fibroblast matrix metalloproteases (MMPs) in response to S. aureus and P. aeruginosa planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM)

In order to determine whether the treatment of equine fibroblasts taken from normal skin and chronic wound granulation tissue, secrete proteases into their surrounding medium following incubation with *S. aureus* and *P. aeruginosa* PCM and

BCM, conditioned medium samples from the *in vitro* scratch assay were run on gelatin zymograms. Controls included DMEM culture medium alone and the conditioned medium from untreated scratches. *S. aureus* and *P. aeruginosa* PCM and BCM alone were also run on the zymogram to determine the proteases that were bacterial-derived. Proteolytic bands of approximately 80kDa and 60kDa were detected in the DMEM and untreated NF and GTF scratch controls (see **Figure 4.5**, **A** and **Figure 4.6**, **A**). Interestingly, the 80kDa band was not present in *P. aeruginosa* PCM and BCM alone and *P. aeruginosa* PCM- and BCM-treated NFs and GTFs.

The bacterial-conditioned medium used in the NF scratch assay showed only a 60kDa protease band (see **Figure 4.5**, **A**). Interestingly, the conditioned medium of NFs treated with *P. aeruginosa* PCM showed the presence of an approximately 57kDa protease band at 48 and 72 hours. The 57kDa protease band was also detected in the conditioned medium of NFs treated with *P. aeruginosa* BCM at 48 and 72 hours, however an additional protease band of approximately 38kDa was also detected at 48 and 72 hours (see **Figure 4.5**, **A**). To see whether the proteolytic bands were metalloproteases, the zymograms were inhibited with EDTA (see **Figure 4.5**, **B**). The 80kDa, 60kDa and 38kDa protease bands were successfully inhibited, however the 62kDa protease present in *P. aeruginosa* BCM alone and *P. aeruginosa* BCM-treated NF conditioned medium remained uninhibited (see **Figure 4.5**, **B**).

Zymography of *P. aeruginosa* PCM and BCM alone, used in the GTF scratch assay, showed the presence of a 62kDa band but also the presence of the 52kDa bacterial-derived band, previously identified in Chapter 3 of this thesis. However another bacterial-derived band of approximately 42kDa was present, as shown by the red box in **Figure 4.6**, **A**. Furthermore, upon treating the zymograms with EDTA, all bands were inhibited except for the bacterial-derived 52kDa and 42kDa protease bands (See **Figure 4.6**, **B**). The treatment of GTFs with *P. aeruginosa* PCM and BCM did not elicit the release of host-derived proteases.

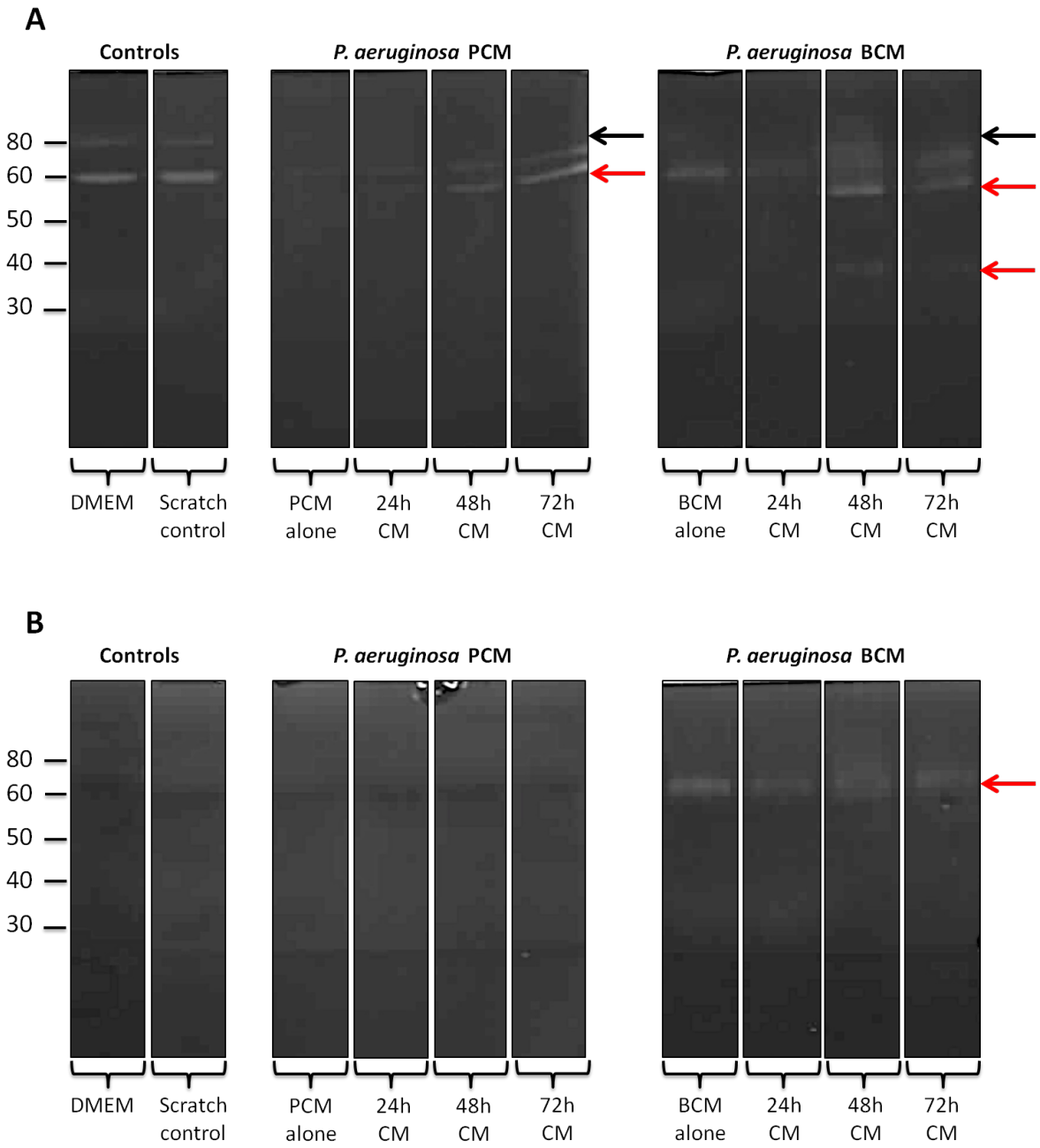


Figure 4.5 Representative zymography of supernatants from the normal fibroblast (NF) scratch wound assay following *P. aeruginosa* PCM and BCM treatment.

Supernatants from *P. aeruginosa* PCM- and BCM-treated cells after 24 and 48 hours were run on gelatin zymograms, which were either untreated (**A**) or inhibited for metalloproteases using 100mM EDTA (**B**). Controls include DMEM culture medium alone and an untreated scratch control. The red arrows indicate the presence of proteases that are of interest and the black arrows indicate the loss of protease band post-treatment. Gelatin zymography of all supernatants from *P. aeruginosa* PCM- and BCM-treated cells were run and repeated twice (technical replicates, n=2).

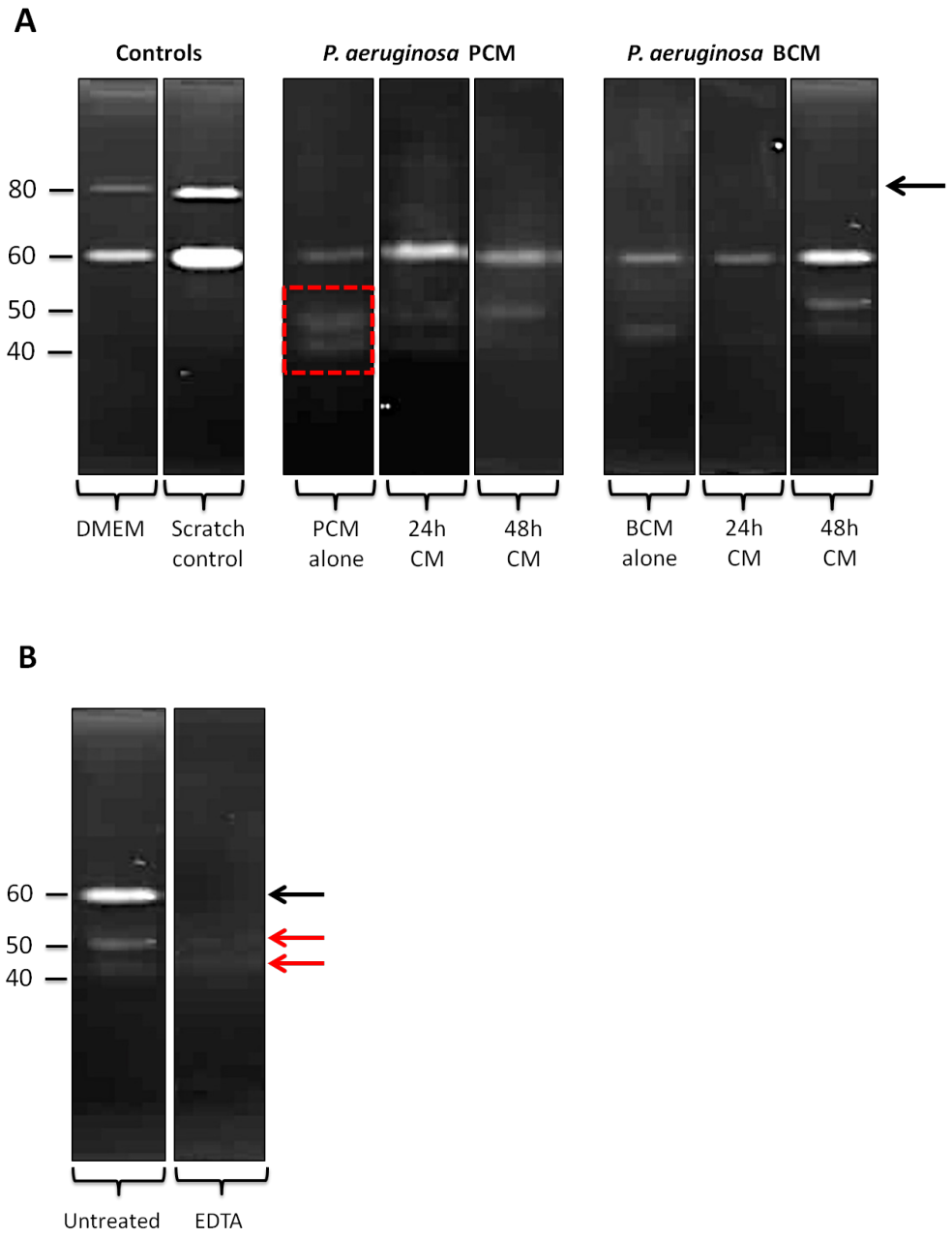


Figure 4.6 Representative zymography of supernatants from granulation tissue fibroblast (GTF) scratch wound assay following *P. aeruginosa* PCM and BCM treatment.

Supernatants from *P. aeruginosa* PCM- and BCM-treated cells after 24 and 48 hours were run on gelatin zymograms, which were either untreated (**A**) or inhibited for metalloproteases using 100mM EDTA (**B**). Controls include DMEM culture medium alone and an untreated scratch control. The red box indicates point of interest. The red arrows indicate the presence of proteases that are of interest and the black arrows indicate the loss of protease band post-treatment. Gelatin zymography of all supernatants from *P. aeruginosa* PCM- and BCM-treated cells were run and repeated twice (technical replicates, n=2).

4.3.3 The expression of matrix metalloproteases (MMPs) in equine normal skin and chronic wound granulation tissue

Normal tissue obtained from the lower leg of horse was stained with haematoxylin and eosin (H&E) to reveal a distinct epidermal layer whereby the basal cell layer (stratum basale), stratum spinosum and the stratum coreum were easily identifiable. The dermis of the normal skin was composed of a network of collagen fibres, which could be seen as pale pink using H&E staining or blue using Masson's trichrome staining. Furthermore, the dermis contained hair follicles, shown by the right-facing arrows (see **Figure 4.7**), which were seen as hollow circular structures surrounded by an epithelial layer. Sebaceous glands were identified near to the base of the hair follicles (see **Figure 4.7**, **A** and **C**). Histological assessment of equine granulation tissue taken from chronic wounds showed a complete absence of an epidermal layer at the wound edge and a large inflammatory cell infiltrate, which was identified by the presence of blue/purple nuclei. In addition, there appeared to be numerous 'pockets' of red staining present in the granulation tissue, which are thought to be the presence of red blood cells. Masson's trichrome staining showed the presence of collagen (pale blue), however the staining was not as strong as the collagen staining in normal skin and there was a distinct lack of collagen fibre organisation. The presence of hair follicles and sebaceous glands were not detected in granulation tissue (see **Figure 4.7**, **B** and **D**).

Normal skin and granulation tissue were treated with a Gram stain to identify the presence of microorganisms within the tissue (see **Figure 4.8**, **B** and **C**). Infected lung tissue was used as a positive control (provided by Veterinary histopathology laboratory, University of Liverpool), clearly showing clusters of Gram-positive bacteria (black) (see **Figure 4.8**, **A**). As expected, the presence of Gram-positive or Gram-negative bacteria was not detected in normal skin. However after thorough microscopic examination, the presence of bacteria in any chronic wound granulation tissue samples were not detected either, contrary to the findings of Westgate and co-workers (Westgate et al. 2011).

MMP-2 antibody staining of normal skin showed strong positive staining in the epidermal layer, specifically the basal cell layer and the stratum spinosum, but also, to a lesser extent, in the epithelial layer surrounding the hair follicles. Slight positive staining was seen in MMP-2-negtive control, particularly in some of the cells of the basal layer and epithelium surrounding the hair follicles. The dermal layer of the normal skin showed faint MMP-2 positive staining that was comparable in intensity to the positive-control stained tissue (see **Figure 4.9**, **A**, **C** and **E**). MMP-2 staining was similar in all normal skin samples assessed. Chronic wound granulation tissue showed faint MMP-2-positive staining throughout the tissue when compared to the controls, however strong staining was identified in an irregular manner, adjacent to the wound edge, although this was not seen in all samples (see **Figure 4.9**, **B** and **D**). Similarly, normal skin sections stained with an MMP-9 antibody showed strong staining in the basal cell layer and stratum spinosum of the epidermal layer and the epithelium surrounding the hair follicles and sebaceous glands, which was comparable to the MMP-9-positive control. The collagen fibres of the dermal layer were not positive for MMP-9 and were comparable to the MMP-9 negative control (see **Figure 4.10**, **A**, **C** and **E**). Chronic wound granulation tissue samples showed MMP-9 positivity ubiquitously throughout the tissue when compared with the negatively stained control section (see **Figure 4.10**, **B** and **D**). MMP-13-positive staining was observed in the epidermal layer of normal skin, with particularly strong staining in the stratum granulosum, the layer of the epidermis directly beneath the stratum corneum, when compared with the negatively stained control. Furthermore, whilst MMP-13 staining was detected throughout the dermal layer, particularly strong staining was seen in the epithelium surrounding the hair follicles and the sebaceous glands (see **Figure 4.11**, **A**, **C** and **E**). In chronic wound tissue, MMP-13-positive staining was ubiquitous when compared to the negatively stained control section, however the intensity of staining was not as strong as the MMP-13-positive control tissue (see **Figure 4.11**, **B**, **D** and **E**).

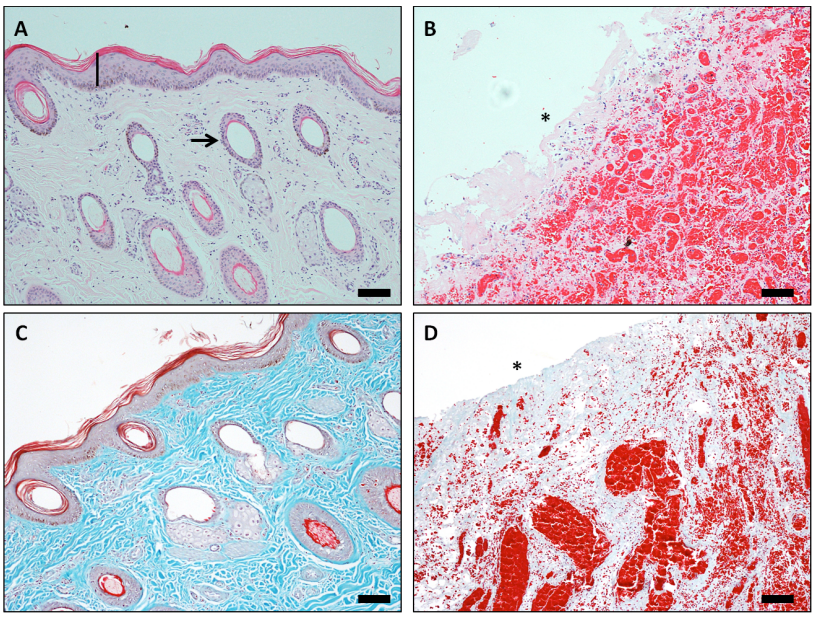


Figure 4.7 Comparison of the basic histological features of normal equine skin (A and C) and equine chronic wound-derived granulation tissue (B and D) stained with haematoxylin and eosin (A and B) and Masson's trichrome (C and D).

Black arrow indicates hair follicle; vertical line highlights the epidermal layer and asterisks (*) indicate the outer wound edge. Images shown are at x10 magnification. Scale bar represents 100µm. Biological replicates, n=8.

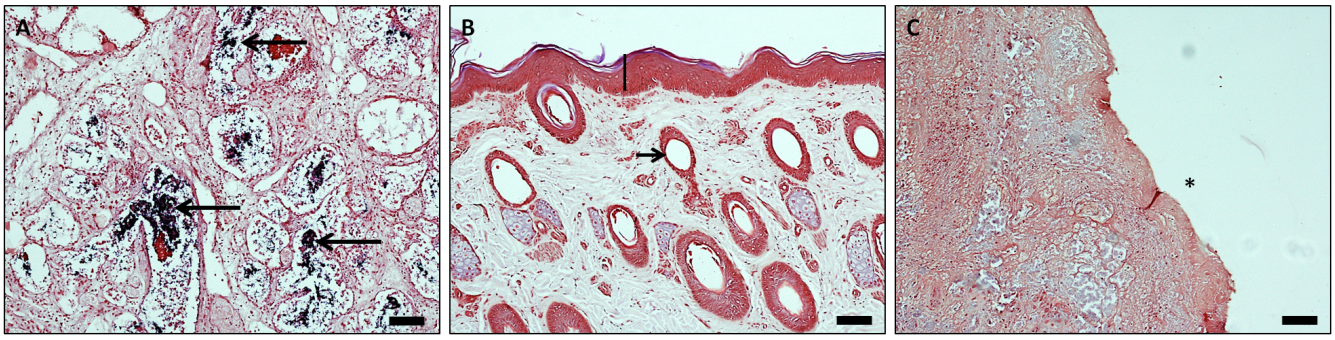


Figure 4.8 Gram staining of normal equine skin (B) and equine chronic wound-derived granulation tissue (C).

For a positive control, chronic infected lung tissue (**A**) was used. Black left-pointing arrows indicate Gram-positive bacterial colonies; black right-pointing arrow indicates hair follicle; vertical line highlights the epidermal layer and the asterisk (*****) indicates the outer wound edge. Images shown are at x10 magnification. Scale bar represents 100µm. Biological replicates, n=8.

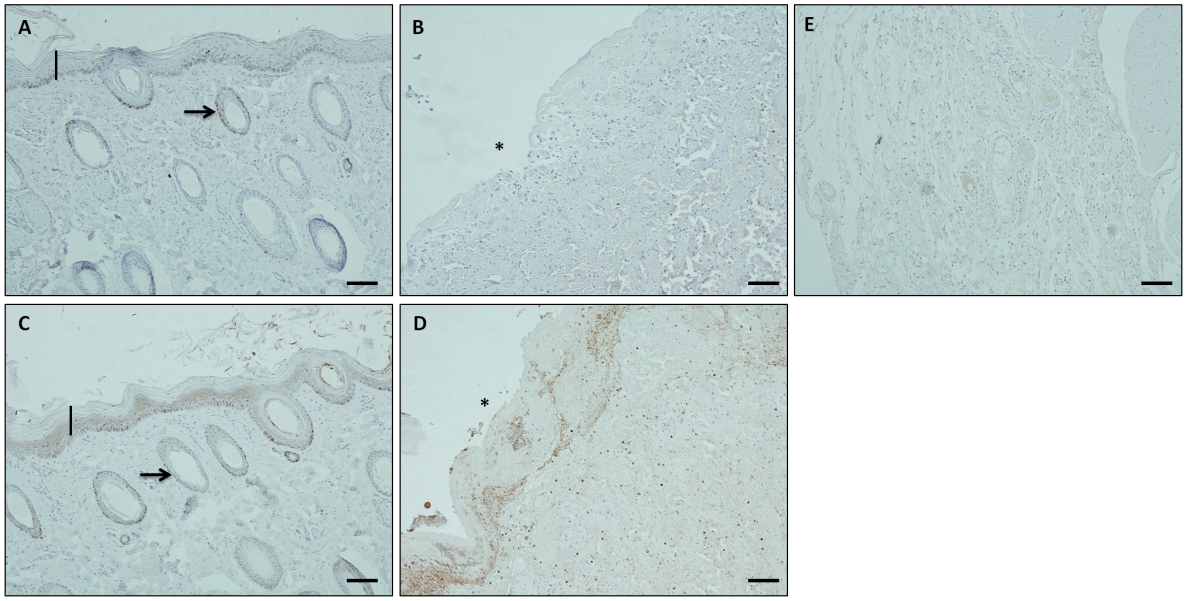


Figure 4.9 The presence of matrix metalloprotease-2 (MMP-2) in equine normal skin (A and C) and chronic wound-derived granulation tissue (B and D).

Tissue from the mammary mass of a cat was used for MMP-2 positive control (**E**). Images (**A**) and (**B**) represent negative staining controls. Black right-pointing arrows indicate hair follicles; vertical line highlights the epidermal layer and the asterisk (*****) indicates the outer wound edge. Images shown are at x10 magnification. Scale bar represents 100µm. Biological replicates, n=8.

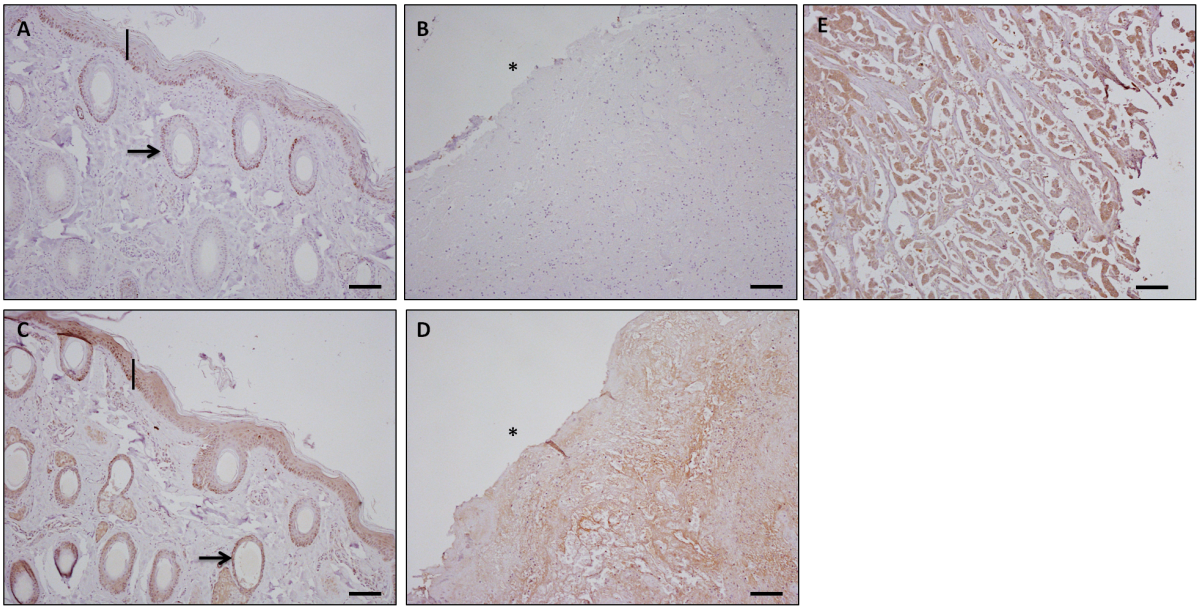


Figure 4.10 The presence of matrix metalloprotease-9 (MMP-9) in equine normal skin (A and C) and chronic wound-derived granulation tissue (B and D).

Tissue from the mammary mass of a cat was used for MMP-9 positive control (**E**). Images (**A**) and (**B**) represent negative staining controls. Black right-pointing arrows indicate hair follicles; vertical line highlights the epidermal layer and the asterisk (*****) indicates the outer wound edge. Images shown are at x10 magnification. Scale bar represents 100µm. Biological replicates, n=8.

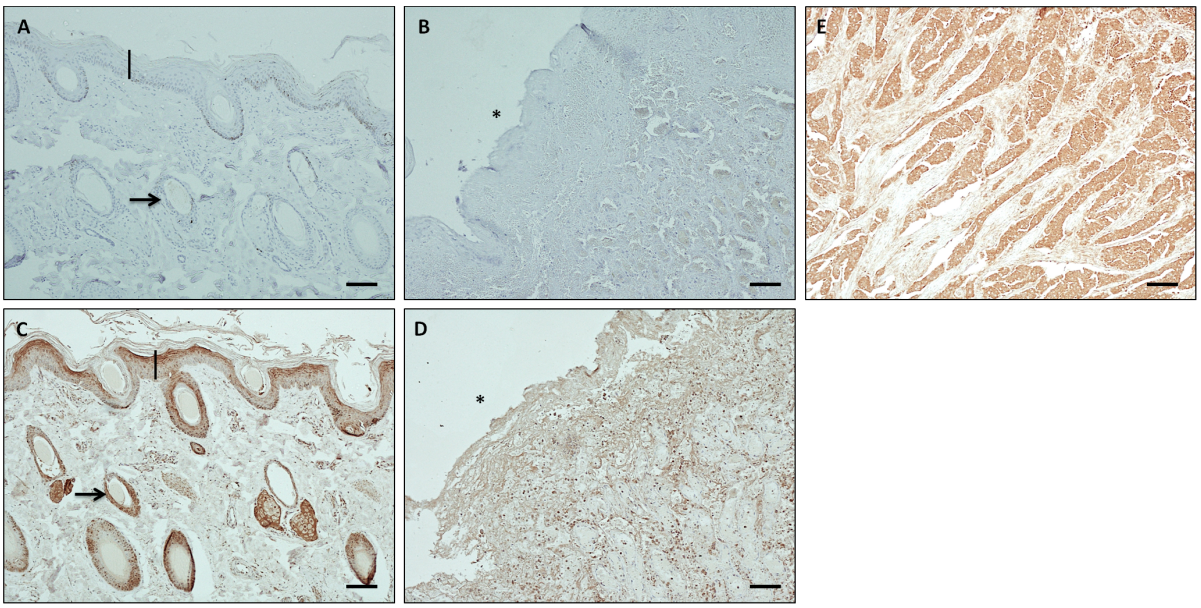


Figure 4.11 The presence of matrix metalloprotease-13 (MMP-13) in equine normal skin (A and C) and chronic wound-derived granulation tissue (B and D).

Tissue from the mammary mass of a cat was used for MMP-13 positive control (**E**). Images (**A**) and (**B**) represent negative staining controls. Black right-pointing arrows indicate hair follicles; vertical line highlights the epidermal layer and the asterisk (*****) indicates the outer wound edge. Images shown are at x10 magnification. Scale bar represents 100µm. Biological replicates, n=8.

4.4 Discussion

One of the aims of this chapter was to assess the effect of bacterial-conditioned medium, in planktonic and biofilm form, on the wound healing of NFs and GTFs, to understand the potential role bacteria plays in equine wound repair. A study by Kirker and colleagues showed a significant decrease in human dermal fibroblast scratch closure when treated with methicillin-resistant *S. aureus* (MRSA) PCM and BCM (Kirker et al. 2012), therefore it was expected that the bacterial-conditioned medium used in this chapter would also reduce scratch closure in equine-derived fibroblasts. Indeed, this study showed a significant reduction in NF and GTF percentage scratch closure upon treatment with *P. aeruginosa* PCM and BCM. This is the first study to demonstrate the effect of bacterial-conditioned medium on equine fibroblast scratch closure.

In this chapter, it appeared that NFs were much more sensitive to treatment with bacterial-conditioned medium than the GTFs. Furthermore, NFs took 72 hours to reach over 95% scratch closure, compared to GTFs, which were able to achieve this closure in 48 hours. The differences noted between the effects of bacterial-conditioned medium, particularly *P. aeruginosa* PCM and BCM, on NFs and GTFs could indicate potential differential effects of bacterial biofilms in acute wounds, where one may expect the presence of normal fibroblasts, and in granulating chronic wounds, where the presence of granulation tissue fibroblasts reside. Therefore, although it is thought that biofilms may lead to the development of human chronic wounds (Bjarnsholt et al. 2008), the presence of biofilms in equine chronic wounds with exuberant granulation tissue, may not have a significant effect on fibroblast migration and viability *in vivo*.

There are a number of studies that have investigated equine wound healing, utilising equine limb fibroblasts (normal fibroblasts). For example Rose investigated the *in vitro* wound closure rates of fibroblasts isolated from equine oral muscosa and equine limb skin in response to various growth factors (Rose 2012). The author found that transforming growth factor beta-1 (TGF-β1), platelet-derived growth factor

(PDGF) and fibroblast growth factor-2 (FGF-2) had differential effects on limb fibroblasts and oral mucosa fibroblasts, potentially providing an insight into the varying clinical outcomes of wounding in these different regions. In the study by Rose, limb fibroblasts showed complete wound closure in 36 hours, in contrast to the 72 hours for NFs in this chapter, however this may be due to the collagen-coated surface in which the fibroblasts were able to migrate across. Furthermore, the method in which the scratch was created in this study differs to the scratch made by Rose, where a vacuum suction and a 200µl pipette tip were used to make a circular wound. Another study, reported increased equine dermal fibroblast migration in response to mesenchymal stromal cell conditioned medium, in an *in vitro* scratch closure model (Bussche et al. 2015), however these cells were from an already established cell line. The isolation and characterisation of fibroblasts from normal skin and the granulation tissue of chronic wounds have been performed before. Cochrane and colleagues assessed the morphology and proliferation rates of both NFs and GTFs and subsequently found that GTFs appeared more cuboidal in morphology, compared to the spindle-like NFs, which support the findings in this chapter. Furthermore, the assessment of proliferation in NFs and GTFs showed faster growth rates in NFs when compared to GTFs (Cochrane et al. 1996), although growth rates were not assessed in this chapter.

In this chapter, zymography was used to detect the presence of proteases in samples of the medium taken at varying time points of the *in vitro* scratch assays. The aim was to determine whether equine-derived fibroblasts from normal skin and granulation tissue secrete proteases in response to the soluble products of bacteria (isolated from wounds) in planktonic and biofilm form. In addition to the bacterial-derived proteases and proteases secreted by the fibroblasts cultured in DMEM (complete), a 57kDa metalloprotease was secreted by NFs in response to *P. aeruginosa* PCM and BCM, but also a 38kDa metalloprotease was secreted from NFs treated with *P. aeruginosa* BCM. Based on the molecular weight and inhibition by EDTA, it is possible that the 57kDa protease is that of the stromelysins, MMP-3 and MMP-10, which both have a molecular weight of 57kDa in their latent enzyme form. However published molecular weights of MMPs that are run on SDS-PAGE may not

have the same molecular weight when run on a gelatin zymogram. Therefore additional studies whereby known MMP-3 and MMP-10 controls are run on the same zymograms would help to confirm this.

Interestingly, no additional and potentially host-derived proteases were detected in the medium of GTFs treated with bacterial-conditioned medium. Given the characteristic over-production of granulation tissue in chronic wounds, these results could suggest a potential dampening in the secretion of MMPs from granulation tissue fibroblasts in the context of the complex environment of equine chronic wounds, leading to an overproduction of extracellular matrix components and a fibroproliferative state. However research into keloid-derived fibroblasts in human has shown an increase in the secretion of active MMP-2 when compared to a normal skin dermal-derived fibroblasts (Imaizumi et al. 2009). Though the use of gelatin zymography alone does not allow the identification of all MMPs, therefore further investigation of other MMPs in this experimental setting using such methods as enzyme-linked immunosorbent assay (ELISA), may give a better understanding into the protease secretome.

An interesting observation was the presence of two protease bands in *P. aeruginosa*-conditioned medium used in the GTF scratch assay, as opposed to the single 52kDa protease band identified in Chapter 3 and in the NF scratch assay. One explanation for this is the use of DMEM to obtain the PCM and BCM. In Chapter 3 of this thesis, bacterial PCM and BCM were obtained using Mueller Hinton broth (MHB), a standard microbiological growth medium. However the use of MHB on mammalian cells in culture caused cell death (data not shown), and therefore it was decided that the use of mammalian cell culture medium, in this case DMEM, would be more appropriate to create bacterial PCM and BCM that could be used to treat the equine fibroblasts. Therefore the change in growth medium could have affected the release of proteases from *P. aeruginosa*. There is however, the matter of differing protease profiles between the *P. aeruginosa*-conditioned medium used in the NF and GTF scratch assay, whereby an additional band of 42kDa was detected in the GTF scratch assay. Indeed, fresh batches of bacterial-conditioned medium were used for the NF and GTF scratch assay, and although the methodology for obtaining the bacterial-

conditioned medium remained the same between batches, changes to the secretion of bacterial proteases may occur after sub-culturing.

The assessment of normal equine skin and chronic wound-derived granulation tissue using basic histology staining and immunohistochemistry was performed in order to assess potential differences in the expression of MMPs between these tissues. Furthermore, following the identification of bacterial biofilms in equine chronic wounds (Westgate et al. 2011), I aimed to discover whether the presence of bacterial biofilms in granulation tissue, affected the expression of MMPs within the surrounding tissue. Surprisingly, in all eight chronic wound-derived granulation tissue samples examined, bacterial biofilms were not identified. Potential reasons as to why these findings do not corroborate the findings of Westgate and colleagues could be due to treatment of the horse prior to obtaining the samples. The criteria for chronic wound sampling in this study included wounds that had not been treated topically by means of cleansing or scrubbing the wound with antimicrobials. However it is possible that treatment with antibiotics could have occurred, which may affect the bioburden of the wound.

Immunohistochemistry showed MMP-2 positive staining was near to the wound edge of the granulation tissue, with faint staining in the surrounding tissue. A significant up-regulation in the expression of MMP-2, TIMP-1 and TIMP-2 mRNA and secreted TIMP-1 in keloid tissue and hypertrophic scars has been recorded in humans, whereby the ratio of MMPs:TIMPs is reduced (Ulrich et al. 2010). Furthermore, the expression of MMP-2 has been shown to be located in the collagen bundles of human keloid tissue (Imaizumi et al. 2009), which may provide a possible explanation for this localisation. Strong MMP-9 staining was detected evenly throughout equine granulation tissue, which has also been demonstrated in human tissue. MMP-9 plays a role in keratinocyte migration in the wound healing process and has been shown to be localised to the epithelium in the oral mucosa, but is also strongly expressed in the granulation tissue of wounded oral mucosa (Salo et al. 1994).

MMP-13 was detected ubiquitously in equine chronic wound-derived granulation tissue. MMP-13 has been documented to regulate the growth of granulation tissue, which has been demonstrated by delayed myofibroblast organisation and granulation tissue growth in MMP-$13^{-/-}$ mice (Toriseva et al. 2012). Furthermore, MMP-13 has also been associated with pathologies such as equine laminitis whereby evidence shows an up-regulation of MMP-13 in the laminae of carbohydrate-overload laminitis, suggesting a role for MMP-13 in extracellular matrix degradation (Wang et al. 2014).

4.4.1 Limitations

There are of course limitations to this study for example; the mechanism by which wound closure is achieved in this study is neither due to migration or proliferation alone, but more likely a combination of both. Therefore the term 'migration' with relation to fibroblast wound closure is avoided. Studies such as those of Rose and Bussche and colleagues, as mentioned earlier, assess fibroblast migration in the *in vitro* scratch assay only after the incorporation of mitomycin C, which effectively inhibits mitosis, eliminating proliferation as a source for wound closure. Albeit the contribution of proliferation towards wound closure in these studies were minimal. Furthermore, the fibroblasts isolated from normal equine skin and granulation tissue were not characterised using antibodies for fibroblasts-specific markers. However, the method used to isolate these fibroblasts is well published (Cochrane et al. 1996) and contaminating cells were not observed throughout culture.

It is important to note that the results obtained from the use of bacterial-conditioned medium to treat fibroblasts may not reflect the outcomes of using planktonic of biofilm bacteria directly within the assay. Whilst the soluble products of bacteria within planktonic and biofilm form clearly affect fibroblast scratch closure, this assay does not account for direct interactions between host cells and bacteria, nor does it account for the effects of host-derived soluble products on bacterial secretions, particular extracellular proteases. Indeed, it was the intention to incorporate bacteria into the assay by means of a 0.2µm cell culture insert, however I

encountered many problems trying to obtain this product as it was not available in most EU countries and shipping from the US proved impossible.

4.4.2 Future work

The use of zymography in this chapter was a good way to identify host-derived secreted proteases, however future work would aim to use more sensitive methods such as enzyme-linked immunosorbent assay (ELISA) to quantitatively assess secreted MMPs. Furthermore the use of reverse transcriptase-polymerase chain reaction (RT-PCR) will aid the understanding of protease gene expression within cells treated with bacterial-conditioned medium. Unfortunately due to technical issues, the detection of TIMPs from equine fibroblasts using reverse zymography was not possible. Given the characteristic excessive granulation tissue formation seen in equine chronic wounds, it would be interesting to understand the role of TIMPs within the wound.

Regarding the *in vitro* scratch wound assay, future work would aim to determine the effect of bacterial-conditioned medium on cell viability and proliferation by using cell viability and proliferation assays such as PrestoBlue™. In addition, repeating these studies on equine keratinocytes may also provide more information on the effects of bacteria on the re-epithelialisation of an equine wound. Advancing upon this, normal equine skin fibroblasts and equine keratinocytes could be used to create a 3-dimensional co-culture skin model *in vitro,* whereby wound closure and MMP expression in the presence of bacterial biofilms could be assessed using histological and immunohistochemistry techniques.

4.4.3 Key points

The PCM and BCM of *P. aeruginosa*, but not *S. aureus*, isolated from equine wounds, caused a significant reduction in the wound closure of NFs and GTFs *in vitro*. Furthermore it appeared that the NF were much more sensitive to *P. aeruginosa*-conditioned medium than the GTFs, although further experiments into cell viability

needs to be performed. Conditioned medium from the *in* vitro scratch assays were assessed for proteases using gelatin zymography. The conditioned medium of NFs treated with *P. aeruginosa* BCM contained 57kDa and 38kDa metalloproteases that were not present in *P. aeruginosa* BCM alone and the NF control medium. There appeared to be no host-derived proteases secreted into the conditioned medium of GTFs treated with bacterial-conditioned medium.

Histological analysis of chronic wound-derived granulation tissue showed a distinct lack of an epidermal layer, hair follicles and sebaceous glands when compared with normal equine skin. Furthermore, there was a lack of collagen fibre organisation in granulation tissue. Surprisingly, the presence of bacterial colonies in granulation tissue samples was not detected. MMP-9 and MMP-13 positive antibody staining was found to be localised in the epidermis and the epithelial cells surrounding hair follicles and sebaceous glands in normal skin, whereas positive staining was ubiquitous throughout the granulation tissue. MMP-2 positive antibody staining was localised to the epidermis in normal skin, however whilst faint staining was seen in the majority of granulation tissue, stronger, more intense MMP-2 staining was identified near to the wound edge.

Chapter 5:

Results III: Protease activity of human chronic wound-derived bacteria

5 Results III: Protease activity of human chronic wound-derived bacteria

5.1 Introduction

Bacterial biofilms in wound healing and the management of chronic wounds are a research field that is generating many questions. This is partly due to the attention that has been drawn to biofilms with regards to their persistence within the host and resistance to antimicrobials within the healthcare setting. A popular example of this is the widely publicised hospital 'super-bug' methicillin-resistant *Staphylococcus aureus* (MRSA), which is thought to reside within a biofilm, which is a common cause of septicaemia or bacteraemia in a clinical setting (HPA 2012). Furthermore, evidence of bacterial biofilms on indwelling medical devices has been documented, such as in the case of ventilator-associated pneumonia (VAP). Vandecandelaere and colleagues identified a diverse range of microorganisms in biofilm form on endotracheal tubes (ETTs), from common oral-associated micro-flora such as *Acinetobacter*, to the more clinically relevant isolates including *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Vandecandelaere et al. 2012).

As mentioned in chapter 1, biofilms have been identified in chronic wounds of humans and other species such as horse and dog, and both *in vitro* and *in vivo* research has shown that the presence of a biofilm can significantly delay wound closure (Kirker et al. 2012, Gurjala et al. 2011) and ultimately retard wound healing. However, one might question whether all biofilms are detrimental to wound healing, or whether is it the presence of biofilms with a predominant pathogenic species of microorganism that leads to impaired wound healing. The demonstration of bacterial biofilms on sutures from both infected and non-infected wounds has highlighted that biofilms can indeed be present at the wound site without causing infection but that there is a difference in microbial profiles associated with clinically diagnosed infection and those with no evidence of infection. Edmiston and colleagues showed *Corynebacterium* to be the most commonly isolated microorganism from the sutures of non-infected wounds, followed by *Bacillus spp.* and *S. epidermidis* (Edmiston et al. 2013). However the microorganisms that dominated sutures from infected wounds

included *S. epidermidis*, *P. aeruginosa,* methicillin-sensitive *S. aureus* and MRSA. Although there was a significant difference in the presence of biofilms in infected wounds when compared to uninfected wounds, 66.6% of uninfected wound-derived sutures were still positive for biofilms. Research by Thomas Bjarnsholt and colleagues in Denmark have advanced the hypothesis that it is the presence of *P. aeruginosa* biofilms that prevents a chronic wound from healing, following research into the pathogenic role of *P. aerguinosa* in cystic fibrosis (Bjarnsholt et al. 2008). *S. aureus* is another microorganism that poses a potential threat to wound healing, as it is a microorganism that can reside asymptomatically on the skin of approximately one third of human beings (Kluytmans et al. 1997). Staphylococcal infection on indwelling medical devices such as catheters is a common problem in the healthcare setting. The potential theory behind this complication identifies the lack of vascularisation at implantation sites, which impedes host defences against colonisation (Kiedrowski and Horswill 2011).

The characteristic tissue degradation often seen in chronic, non-healing wounds brought about the theory of a potential dysregulation in host protease production (Velnar et al. 2009). Indeed, this theory was addressed and subsequent studies in the 1990's revealed significantly increased levels of specific MMPs in chronic wound fluid (Wysocki et al. 1993, Nwomeh et al. 1999). More specifically, significant increases in the gelatinases MMP-2 and MMP-9 in chronic wound fluid, has been a common finding in a variety of non-healing chronic wounds (Nwomeh et al. 1999, Yager et al. 1996, Wysocki et al. 1993). In these studies, and indeed in many studies investigating host proteases, the method of zymography is used, whereby a substrate is incorporated into a polyacrylamide gel in order to determine the molecular weight of the protease that degrades that particular substrate within the gel. This method of protease detection is not specific to mammalian-derived proteases and is also a method used to identify many bacterial-derived proteases (Lantz and Ciborowski 1994). Therefore with reference to these earlier studies, it is interesting to consider whether other proteolytic enzymes found in chronic wound fluid were indeed bacterial-derived, and thus contributing to the high levels of proteases seen in many chronic wounds today.

Despite the already well-documented altered proteolytic environment of a chronic wound and the more recent implications of bacterial biofilms, the contribution of bacterial-derived extracellular proteases in chronic wounds has been poorly researched. One only has to enter 'bacterial proteases AND chronic wounds' into the PubMed search engine to reveal a mere 43 results, of these, only 2 primary research papers specifically address bacterial proteases in the context of chronic wounds (Schmidtchen et al. 2001, Wysocki et al. 2012). These studies typically assess the proteolytic activity of bacteria grown in a planktonic or 'free-floating' state. For example, Wysocki and colleagues assessed bacterial gelatinase activity using gelatin-agarose gel in which they diluted a single bacterial colony in HEPES buffer and directly incubated with the gel (Wysocki et al. 2012). There is currently very little evidence of the proteolytic activity of human chronic-wound-derived bacteria when grown in a biofilm state. However, one study touched upon this area with respect to antimicrobial resistance and bacterial virulence, when questioning whether *P. aeruginosa* protease production was affected after antibiotic treatment. Here it was found that extracellular proteases secreted from two burn wound-derived strains of *P. aeruginosa,* in biofilm form, secreted elastase B (LasB) post-ciprofloxacin treatment, although total proteolytic activity was reduced (Ołdak and Trafny 2005). Given the increased antimicrobial resistance of bacterial biofilms, this research highlighted the persistence and virulence of those biofilm-derived cells that remain unaffected by antimicrobials.

Given that some of the most commonly isolated bacteria from human chronic wounds include *S. aureus* (93.5%) and *P. aeruginosa* (52.2%) (Gjødsbøl et al. 2006), it was the objective of this chapter to assess the proteolytic activity of human chronic wound-derived *P. aeruginosa* and *S. aureus* in both planktonic and biofilm states, followed by the identification of these proteases. More specifically, clinical isolates were used to produce planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM), which were subjected to non-specific proteases assays in order to assess protease activity and zymography to detect proteases and determine the molecular weight/protease group. Based on the findings in Chapter III of this thesis, I hypothesised that *P. aeruginosa* in planktonic and biofilm form would

secrete high levels of proteases and that *S. aureus* would produce low or undetectable levels of proteases.

5.2 Methods

The bacterial isolates used in this chapter were derived from human chronic wounds, including leg ulcers and surgical wounds (see **Table 5.1**), and were obtained from Professor David Williams of Cardiff University, UK. Ethical approval for the collection of these isolates from patients was approved as part of a separate research project at Cardiff University. For a detailed description of the methods used in this chapter, the reader is directed to Chapter 2 of this thesis (2.3 Microbiology, 2.3.6 Crystal Violet Biofilm Forming Assay, 2.5 Scanning Electron microscopy, 2.6 Non-specific Protease Assays, 2.7 Gel Electrophoresis, 2.8 Ammonium Sulfate Precipitation and Partial Purification of Proteins, 2.9 BCA Assay and Sample Protein Quantification and 2.18 Statistical Analysis).

5.3 Results

5.3.1 Growth curve analysis and biofilm forming potential of human chronic wound-derived P. aeruginosa and S. aureus

The growth curves in this experiment were performed in a 96-well format whereby the absorbance of each well was measured at several time points (every 4 hours) over a 72-hour period. Within each bacterial species, both *P. aeruginosa* and *S. aureus* isolates followed similar growth curve profiles. However two *P. aeruginosa* isolates, P1139 and P1145, showed a greater amount of proliferation when compared to the other *P. aeruginosa* isolates (see **Figure 5.1, A**). All isolates entered the exponential phase between 0-hours and 4-hours, which reached its peak between 8 and 12 hours. After this, growth curves for all isolates then reached a plateau (stationary phase) that was either maintained throughout the course of the experiment, which was seen in the *P. aeruginosa* isolates (see **Figure 5.1, A**), or

maintained for a short time period before a decline in the growth curve (cell death), which was seen more in the *S. aureus* isolates (see **Figure 5.1, B**).

Biofilms are described as communities of microorganisms that attach to a surface or each other, proliferate and surround themselves in an EPS matrix. Scanning electron microscopy (SEM) is a regularly utilised method of biofilm identification, as it allows for the identification of biofilm-specific morphology and the spatial location of individual cells at a high resolution (Oates et al. 2014). In this experiment, biofilms were grown on sterile 0.2µm membrane filter discs for 72 hours. Upon washing the biofilms prior to fixation, it was noted that a large proportion of *S. aureus* was easily washed away from the membrane filter and, although some *P. aeruginosa* was removed from the membrane filter upon washing, the *P. aeruginosa* biofilm remained relatively intact. This observation was substantiated upon SEM imaging of the biofilms. It was evident that clinically derived *P. aeruginosa* formed a dense biofilm, with *P. aeruginosa* rods barely visible under what was believed to be an EPS matrix (see **Figure 5.3, B**). The clinical *S. aureus* showed communities of spherical cocci; however these where scant when compared to the thick biofilm produced by clinical *P. aeruginosa* (see **Figure 5.3, A**). Following this, the biofilm forming potential of *S. aureus* and *P. aeruginosa* isolates was evaluated by staining 24, 48 and 72 hour biofilms produced in a 96-well peg-lid plate format, with crystal violet. In this assay, the crystal violet dye, which appears dark purple in colour, binds to the adherent bacteria before being solubilised and measured using absorbance. High absorbance values indicate high numbers of adherent bacterial cells, due to the intensity of dye in the well, and thus strong biofilm formation (George 2011). In a similar fashion, low absorbance values (low intensity of stain in the well) indicate low numbers of adherent bacterial cells, and thus poor biofilm formation (see **Figure 5.2, C**). In this study, the majority of clinical *S. aureus* isolates showed low biofilm forming potential which did not significantly alter between 24, 48 and 72 hours. However some anomalies with much higher biofilm forming potential were recorded such as the clinical isolates S1007, S1010 and S1070 (see **Figure 5.2, A**).

Although it was the primary aim of this chapter to assess differences between bacterial isolates, bacterial isolates were taken from a variety of wounds including

chronic and surgical wounds (see **Table 5.1**). Therefore it is important to note that there appeared to be no correlation between the higher biofilm forming potential phenotype and the wound type. There were no significant differences in the mean absorbance values between *S. aureus* biofilms at 24, 48 and 72 hours. Clinical *P. aeruginosa* isolates showed varied biofilm forming potential and, although the strongest biofilm forming potential appeared to be at 72 hours, variability in absorbance values between each isolate resulted in no significant differences between 24, 48 and 72-hour biofilms.

Strain	Isolate Code	Type of Wound	Age of Patient	Gender
P. aeruginosa	P1006a	Leg ulcer	56	F
P. aeruginosa	P1007a	Leg ulcer	85	F
P. aeruginosa	P1097a	Surgical wound	85	F
P. aeruginosa	P1102a	Venous leg ulcer	59	M
P. aeruginosa	P1120a	Venous leg ulcer	50	M
P. aeruginosa	P1126a	Leg ulcer	90	F
P. aeruginosa	P1131a	Leg ulcer	61	F
P. aeruginosa	P1134a	Surgical wound	76	F
P. aeruginosa	P1139a	Leg ulcer	78	F
P. aeruginosa	P1140a	Leg ulcer	65	M
P. aeruginosa	P1145a	Foot ulcer	69	F
P. aeruginosa	P1146a	Venous leg ulcer	66	F
P. aeruginosa	P1147a	Leg ulcer	79	F
P. aeruginosa	P1151a	Leg ulcer	93	F
P. aeruginosa	P1152a	Foot ulcer	66	M
S. aureus	S1002a	Leg ulcer	63	F
S. aureus	S1003a	Venous leg ulcer	47	M
S. aureus	S1006a	Leg ulcer	56	F
S. aureus	S1007c	Leg ulcer	85	F
S. aureus	S1010a	Venous leg ulcer	67	M
S. aureus	S1012a	Venous leg ulcer	57	M
S. aureus	S1014a	Surgical wound	54	M
S. aureus	S1016	Venous leg ulcer	60	F
S. aureus	S1019a	Venous leg ulcer	76	F
S. aureus	S1030a	Surgical wound	52	M
S. aureus	S1059a	Leg ulcer	50	F
S. aureus	S1060a	Surgical wound	75	M
S. aureus	S1068a	Venous leg ulcer	49	M
S. aureus	S1069a	Foot ulcer	38	M
S. aureus	S1070a	Leg ulcer	39	F
S. aureus	S1071b	Leg ulcer	65	M
S. aureus	S1078a	Surgical wound	52	F
S. aureus	S1087a	Leg ulcer	81	M
S. aureus	S1095a	Leg ulcer	72	F

Table 5.1 Origin of human-derived bacterial isolates.

Bacterial isolates obtained from Cardiff University). Abbreviations include F = Female, M = Male, *S. aureus* = *Staphylococcus aureus*, *P. aeruginosa* = *Pseudomonas aeruginosa*.

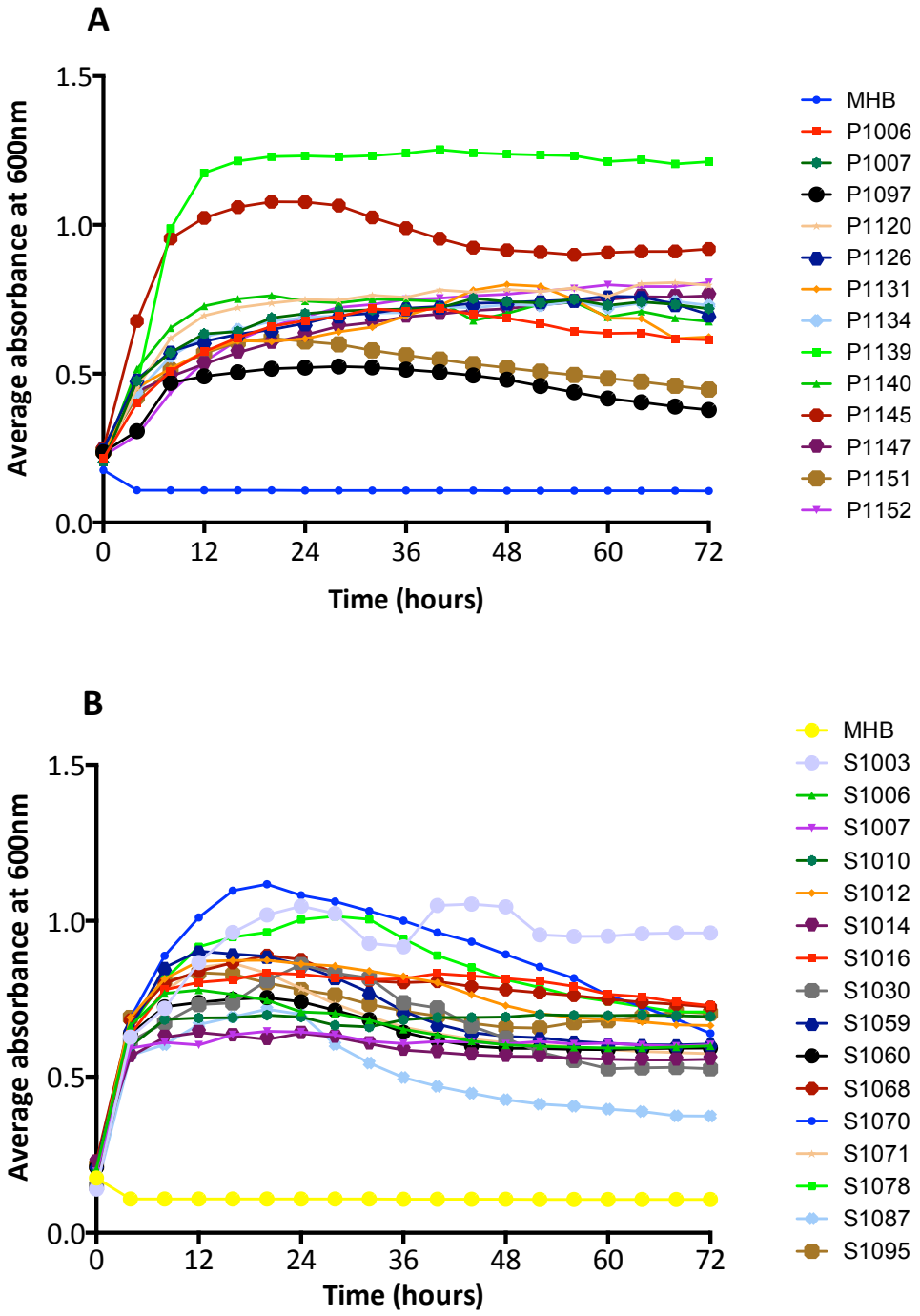
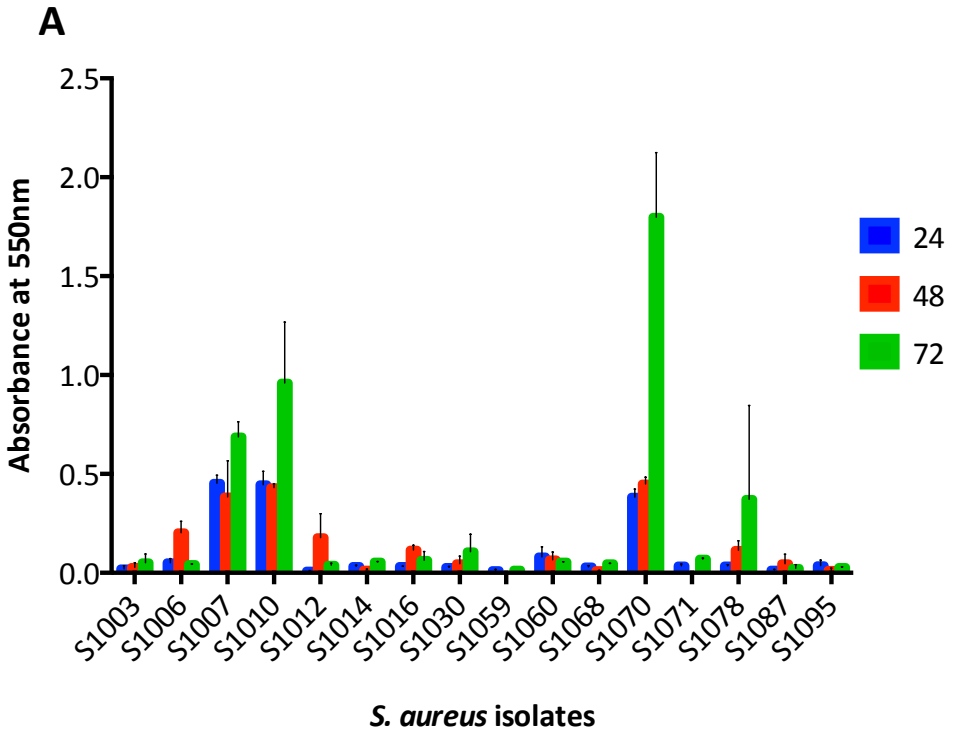


Figure 5.1 Growth curves of *P. aeruginosa* and *S. aureus* over 72 hours.

P. aeruginosa (**A**) and *S. aureus* (**B**) clinical isolates were individually seeded into a 96-well plate and incubated for 72 hours, whereby the absorbance of each well containing the isolate was measured every 4 hours. Mueller Hinton broth (MHB) was used as a negative (no growth) control. The assay was repeated three times (technical repeats) and the average absorbance values obtained and used to plot the growth curves.


A
Absorbance at 550nm
2.5
2.0
1.5
1.0
0.5
0.0
24
48
72
S1003
S1006
S1007
S1010
S1012
S1014
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S1059
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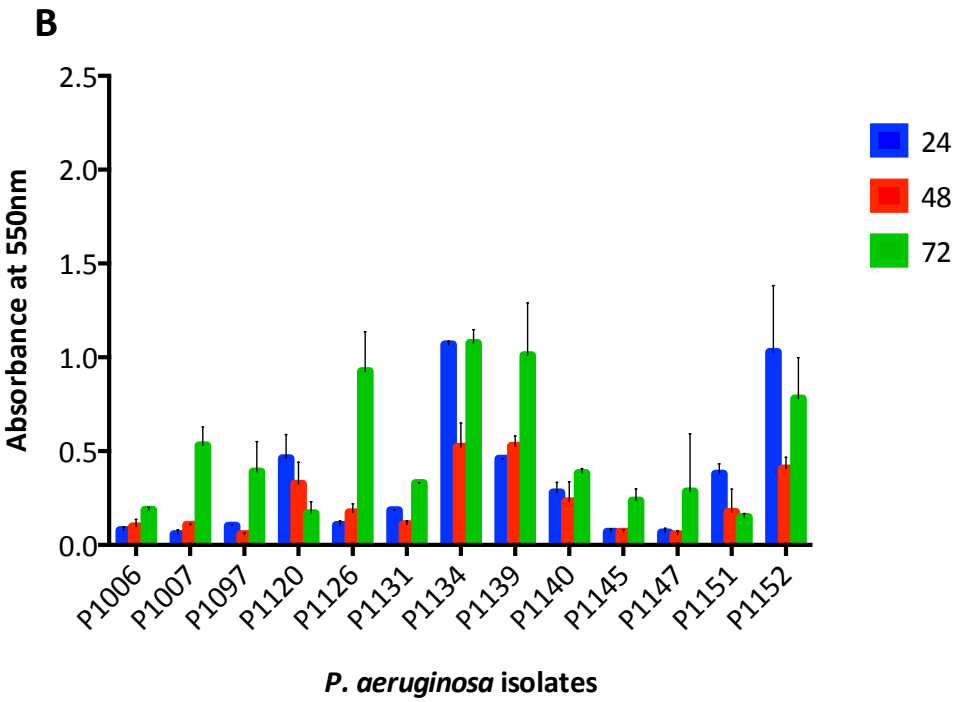
S1071
S1078
S1087
S1095
S. aureus isolates


B
Absorbance at 550nm
2.5
2.0
1.5
1.0
0.5
0.0
24
48
72
P1006
P1007
P1097
P1120
P1126
P1131
P1134
P1139
P1140
P1145
P1147
P1151
P1152
P. aeruginosa isolates

C

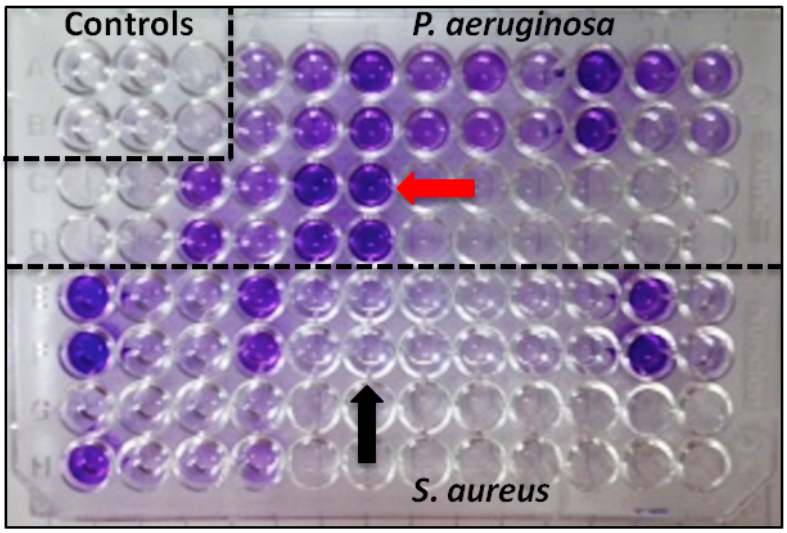


Figure 5.2 Biofilm forming potential of human-derived clinical isolates using crystal violet.

S. aureus (**A**) isolates and *P. aeruginosa* (**B**) isolates were incubated for 24, 48 or 72 hours before being stained with crystal violet, solubilised and read at a wavelength of 550nm. The crystal violet assay plate (**C**) shows the solubilised crystal violet from 72-hour biofilms. The red and black arrow indicates strong and weak biofilm forming potential respectively. Controls include Mueller Hinton broth (MHB) without microorganisms (negative control). The assay was repeated three times with all clinical isolates. Error bars represent standard deviation.

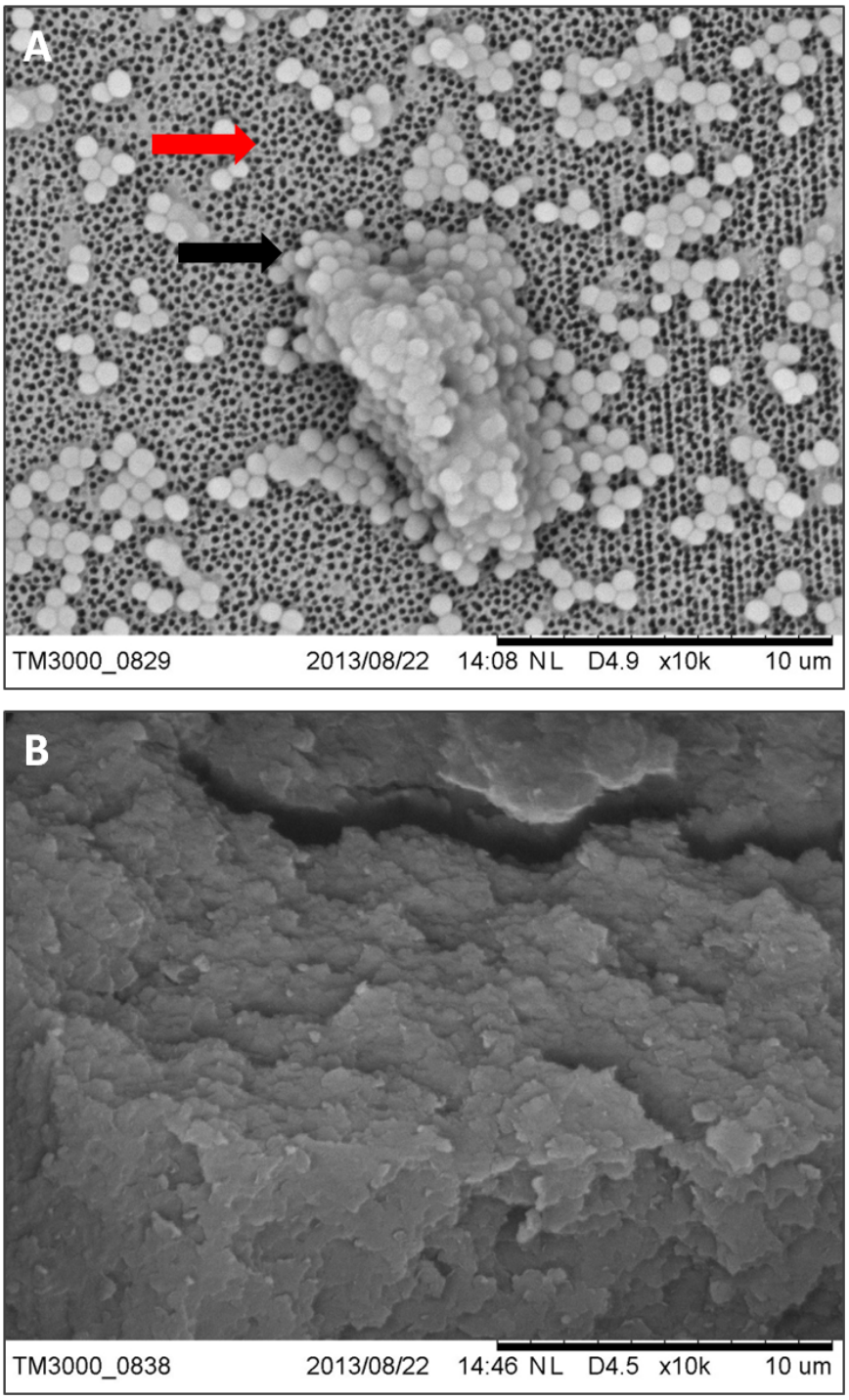


Figure 5.3 Scanning electron microscopy (SEM) of 72-hour human-derived bacterial biofilms grown on membrane filter discs.

A. *S. aureus* and **B**. *P. aeruginosa* were grown on membrane filter discs for 72 hours before being washed in PBS and fixed in 2.5% gluteraldehyde and dehydrated in ethanol. Images were taken using the Hitachi TM300 tabletop scanning electron microscope. Magnification 10,000x. Black arrow indicates bacterial clustering, red arrow shows the membrane filter surface.

5.3.2 Proteolytic activity of human chronic wound-derived P. aeruginosa and S. aureus in planktonic and biofilm form

The assessment of proteolytic activity using the Azo-casein assay revealed a significant difference in the proteolytic activity of *P. aeruginosa* BCM when compared with *P. aeruginosa* PCM *($P \leq 0.0001$)*, which produced low levels of proteases. Furthermore, significant differences were found between *P. aeruginosa* BCM and *S. aureus* BCM *($P \leq 0.05$),* with *S. aureus* showing equally low proteolytic activity in the PCM and BCM (see **Figure 5.4**). The Azocoll assay revealed similar results to the Azo-casein assay, showing low proteolytic activity in both *S. aureus* and *P. aeruginosa* PCM. *S. aureus* BCM showed no increase in proteolytic activity when compared to *S. aureus* PCM, however a significant increase in activity was recorded for *P. aeruginosa* BCM when compared to *P. aeruginosa* PCM *($P \leq 0.0001$)* and *S. aureus* BCM *($P \leq 0.05$)* (see **Figure 5.5**). Although the majority of *P. aeruginosa* BCM biological replicates showed high proteolytic activity, there was still variability in activity across biological replicates with some isolates showing lower activity than expected.

Traditionally, collagen zymography is used to identify collagenases such as MMP-1, MMP-9 and MMP-13 (Snoek and Von den Hoff 2005). However, as summarised in chapter 1 of this thesis (see **Table 1.1**), a wide range of MMP's can actively breakdown collagen as a substrate. Therefore, in order to determine what proteases were being produced by *P. aeruginosa* in biofilm-form, BCM samples from all isolates were subject to collagen zymography. In this experimental setting, a positive control of equine-derived MMP-2 was loaded on the collagen zymogram, which resulted in a 68kDa pro-enzyme band and a 58kDa active band respectively. In addition, a DMEM culture medium control sample was loaded to represent the medium by which the biofilm was suspended. The DMEM control sample showed faint proteolytic bands of approximately 80kDa and 68kDa. Interestingly the clinical *P. aeruginosa* BCM samples showed strong proteolytic bands at approximately 62kDa and 52kDa. The presence of the 62kDa band was seen in the majority of the BCM samples, however a smaller number of isolates expressed both the 62kDa and 52kDa band simultaneously (see **Figure 5.6**), highlighting the variability of proteolytic activity

between the various clinical strains of *P. aeruginosa*. There was no correlation between the presence of the 52kDa band and wound type.

In order to assess which of the major proteolytic groups these *P. aeruginosa*-derived proteases belong to, a clinical isolate which was shown to produce proteases was run separately on a collagen zymogram and subsequently incubated with broad spectrum inhibitors of either metallo-, serine or cysteine proteases (EDTA, benzaminide hydrochloride hydrate and N-ethylmaleimide (NEM)). The treatment of the proteolytic bands with EDTA, benzamidine and NEM revealed that the treatments were ineffective in the inhibition of the proteolytic bands (see **Figure 5.7**).

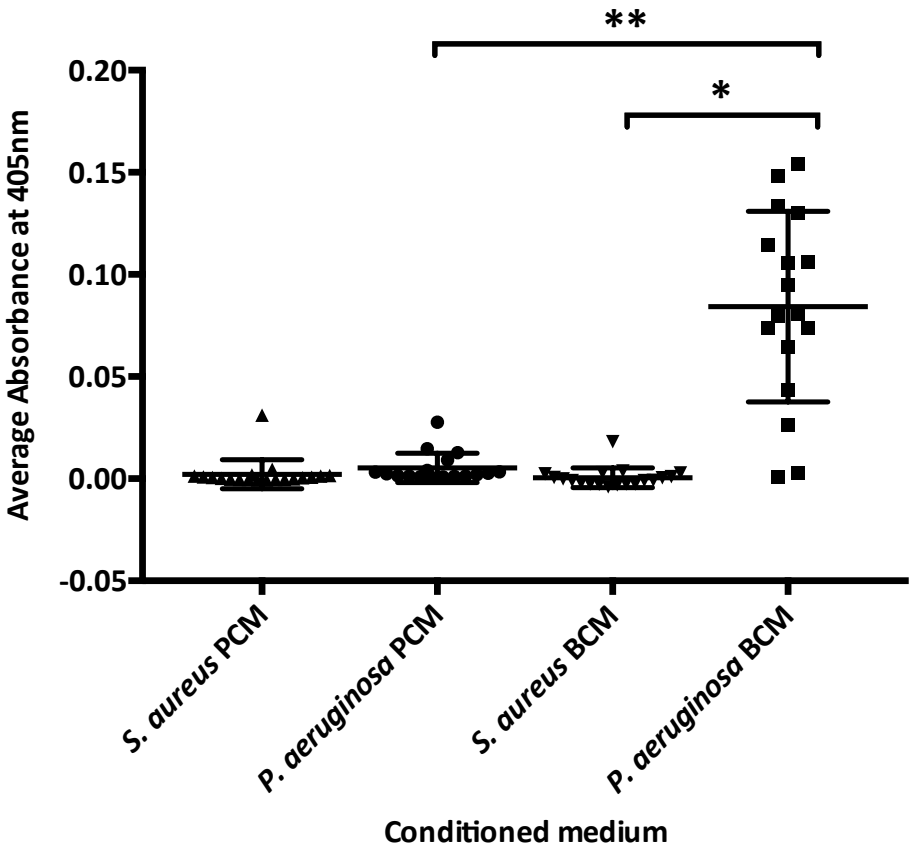


Figure 5.4 The non-specific proteolytic activity of human-derived *S. aureus* and *P. aeruginosa* clinical isolates using the Azo-casein assay.

Data from the biological replicates were combined in order to assess the statistical difference of average absorbance values between clinical *S. aureus* and *P. aeruginosa*. All biological replicates in the assay were run in duplicate. Error bars represent standard deviation. Statistical analysis was performed using the Kruskal-Wallis non-parametric ANOVA, with Dunn's multiple comparison test; *P < 0.05, **P < 0.0001. Abbreviations include planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

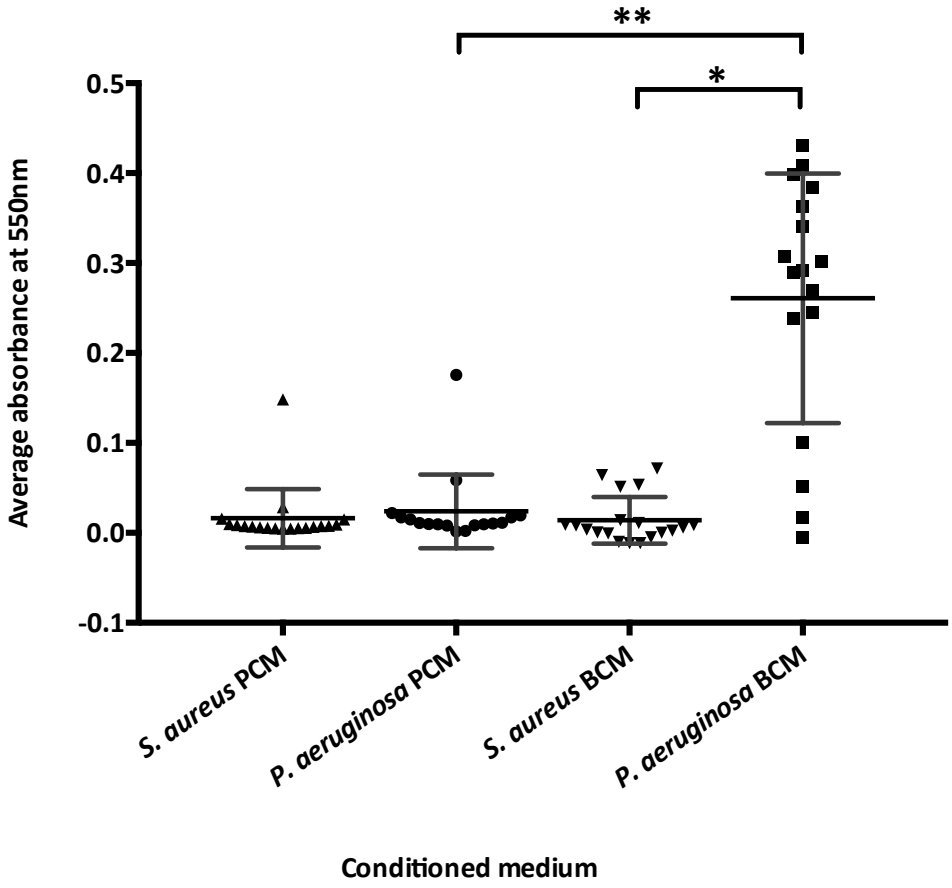


Figure 5.5 The non-specific proteolytic activity of human-derived *S. aureus* and *P. aeruginosa* clinical isolates using the Azocoll assay.

Data from the biological replicates were combined in order to assess the statistical difference of average absorbance values between clinical *S. aureus* and *P. aeruginosa*. All biological replicates in the assay were run in duplicate. Error bars represent standard deviation. Statistical analysis was performed using the Kruskal-Wallis non-parametric ANOVA, with Dunn's multiple comparison test; *P < 0.05, **P < 0.0001. Abbreviations include planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

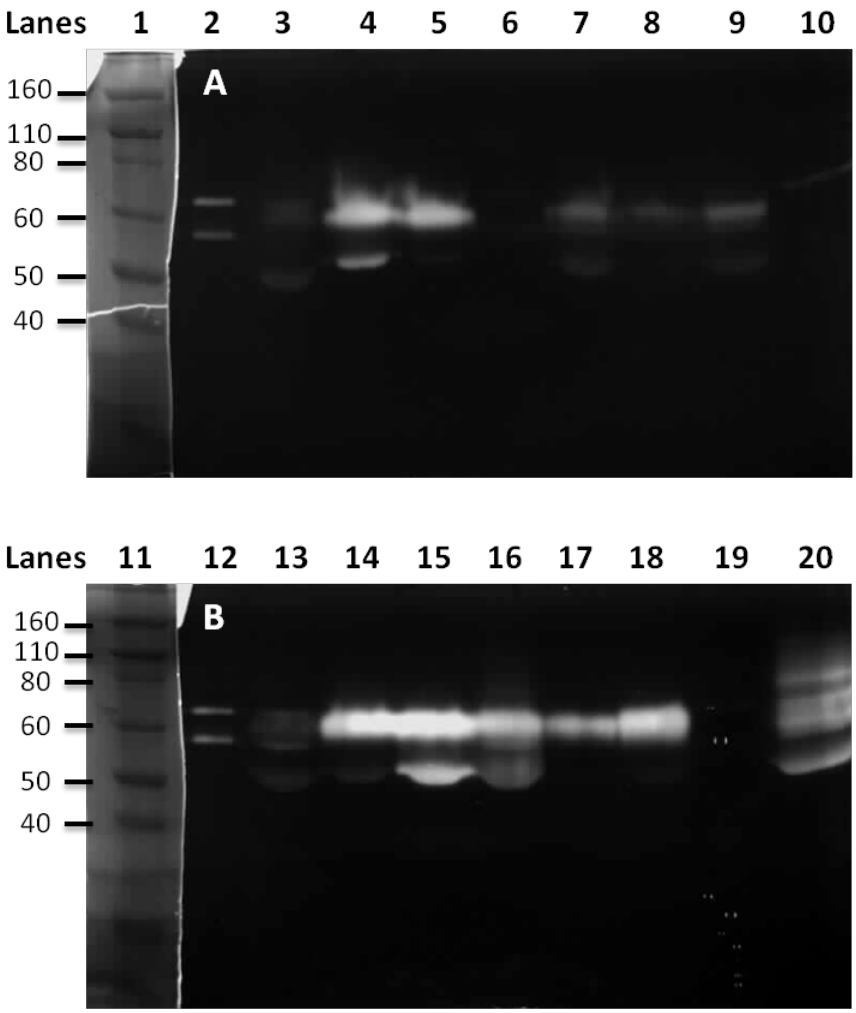


Figure 5.6 Collagen zymography of human-derived *P. aeruginosa* biofilm-conditioned medium (BCM).

Lanes 3-9 (**A**) represent clinical isolates P1006-P1131 and lanes 13-20 (**B**) represent clinical isolates P1134-P1152 (see **Table 5.1** for reference). Lanes 1 and 11 represent the molecular weight ladder (labelled in kDa). Controls include equine fibroblast-derived MMP-2 (lanes 2 and 12) and DMEM culture medium (lane 10). The experiment was performed using all biological replicates of *P. aeruginosa* (n=15) and was repeated three times.

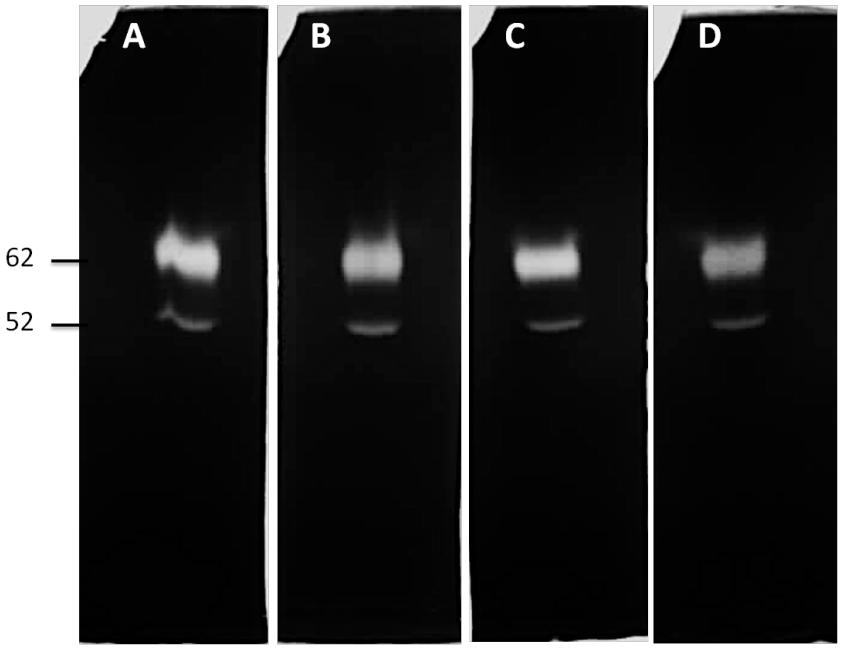


Figure 5.7 The effect of broad-spectrum protease inhibitors on human-derived *P. aeruginosa* proteases detected in BCM.

Clinical isolate P1097 BCM was run on a collagen zymogram before cutting the zymogram into sections containing the sample and incubating in either (**A**) Normal, untreated buffer, (**B**) 100mM ethylenediaminetetraacetic acid (EDTA)-treated buffer. (**C**) 50mM benzamidine hydrochloride hydrate-treated buffer or (**D**) 10mM N-ethylmaleimide (NEM)-treated buffer.

5.3.3 Partial protease purification of P. aeruginosa extracellular proteases

Precipitation is a commonly used method in the recovery of biomolecules such as proteins from complex protein samples. In this chapter, ammonium sulfate (AS) ($(NH_4)_2SO_4$) precipitation was used in the attempt to purify the bacterial proteases detected in zymography. AS precipitation works by interfering with the hydrophilic amino acid region on the protein and water, effectively reducing the solubility of the protein and encouraging hydrophobic interactions, leading to the aggregation of the protein from solution. Proteins have different proportions of hydrophilic regions,

thus making it possible to separate particular proteins based on their hydrophilicity. For example, a protein with relatively few hydrophilic regions will aggregate at low concentrations of AS and a protein with many hydrophilic regions will need a much higher concentration of AS to precipitate from the solution (Scopes 1994).

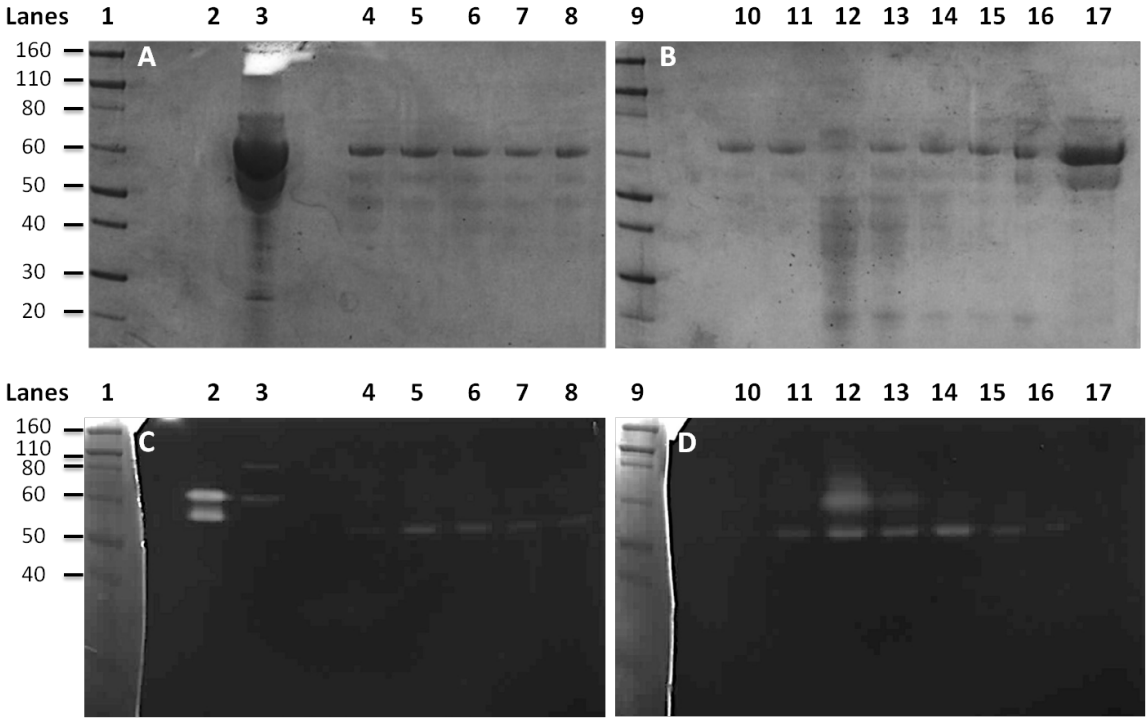
In order to investigate the role of human chronic wound-derived *P. aeruginosa* 62kDa and 52kDa proteases in wound healing, partial purification of BCM was performed using AS precipitation followed by dialysis in PBS. The dialysed AS fractions were then subject to SDS-PAGE and zymography to evaluate the protein and enzyme profile of each fraction. The MMP-2 positive control showed clear proteolytic bands at approximately 68kDa and 58kDa. However no bands were detected in SDS-PAGE (see **Figure 5.8**; **A** and **C**, lane 2). The DMEM culture medium control showed a dense protein profile that was particularly saturated between the molecular weights of 50-60kDa, and as well as further proteolytic bands at 60kDa and 80kDa (see **Figure 5.8**; **A** and **C**, lane 3). In all precipitate fractions there was a 62kDa band shown in the SDS-PAGE gels (see **Figure 5.8**; **A** and **B**, lanes 4-8 and 10-16), however, only the 50% and 55% fractions displayed 62kDa proteolytic bands (see **Figure 5.8**; **C** and **D**, lanes 12 and 13). In addition to this, the 50% and 55% fractions showed the highest protein concentrations of 835.1µg/ml and 490.7 µg/ml respectively (see **Table 5.2**). Furthermore, in 11 of 12 precipitate fractions there was the presence of a 52kDa proteolytic band, of varying intensities, seen in collagen zymography; however it was difficult to detect the presence of this band using SDS-PAGE (see **Figure 5.8**; **A**, **B**, **C** and **D**, lanes 4-8 and 11-16). The 50% and 55% precipitate fractions were the only fractions containing both the 52kDa and 62kDa proteolytic bands (see **Figure 5.8**; **D**, lanes 12 and 13). There were no proteolytic bands evident in the 40% fraction (see **Figure 5.8**; **D**, lane 10). Following the 70% AS precipitation of the BCM, the remaining supernatant was collected and run on the SDS-PAGE gel and collagen zymogram. It was clear from zymography that the desired proteolytic bands were not present in the remaining supernatant (see **Figure 5.8**; **D**, lane 17). Additionally, protein fractions were subject to the Azo-casein and Azocoll assays. The Azo-casein assay revealed a significant level of proteolytic activity in the 50% protein fraction when compared to the PBS control ($P < 0.05$) (see **Figure 5.8, E**).

The Azocoll assay showed high levels of protease activity in all fractions, however statistical analysis showed the 50% protein fraction to have significantly high protease activity when compared to the PBS control (see **Figure 5.8, F**). Furthermore, this high protease activity appeared to reflect the high protein concentration in this fraction, as determined by the BCA assay (see **Table 5.2**). It was therefore concluded that 50% ammonium sulfate precipitation would be suitable to isolate the 62kDa and 52kDa proteases from human chronic wound-orginating *P. aeruginosa* BCM.

Starting volume (ml)	**Ammonium sulfate saturation (%)**	**Amount of $(NH_4)_2SO_4$ added (g)**	**Protein concentration post-dialysis (µg/ml)**
11.8	15	0.96	266.8
11.8	20	0.32	231.2
11.2	25	0.31	202.1
10.7	30	0.30	158.4
10.4	35	0.30	197.3
9.9	40	0.29	172.8
9.4	45	0.28	206.8
9.1	50	0.27	835.1
8.6	55	0.26	490.7
8.2	60	0.26	286.0
8.0	65	0.25	248.7
7.6	70	0.24	328.7

Table 5.2 Multiple protein fractions of P1097 BCM using ammonium sulfate precipitation and the subsequent protein concentration of the dialysed protein fractions.

The concentration of protein at the various ammonium sulfate saturation percentages was performed using the BCA protein assay. The chemical formula for ammonium sulfate is written as $(NH_4)_2SO_4$.


Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
160
110
80
60
50
40
30
20
A
B
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
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110
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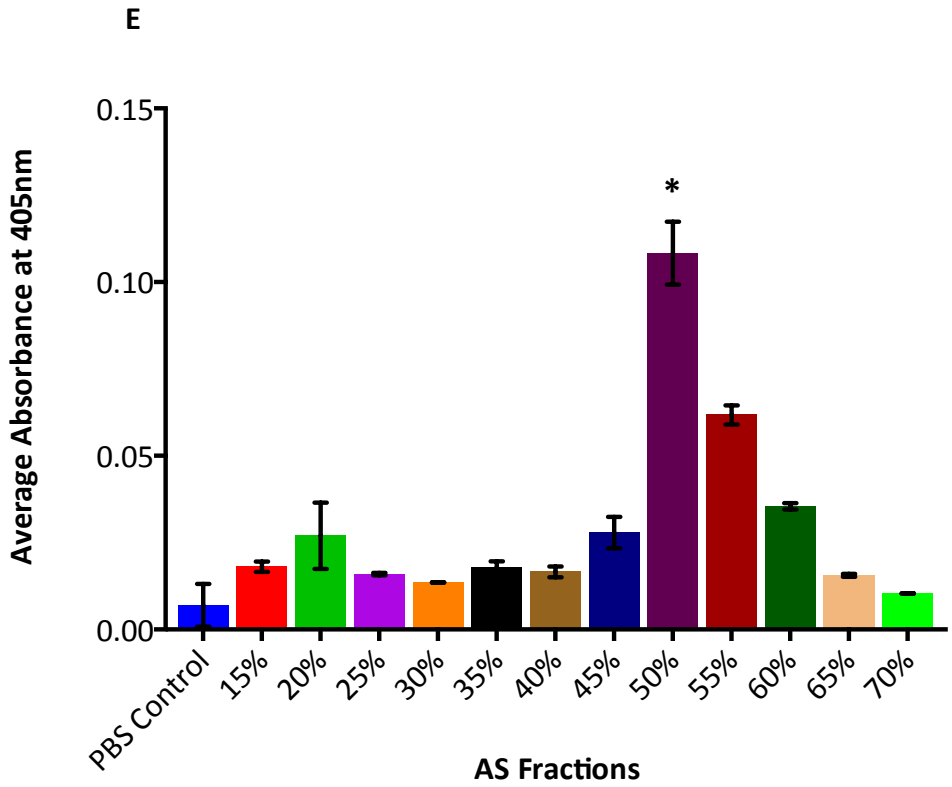
60
50
40
C
D


E
Average Absorbance at 405nm
0.15
0.10
0.05
0.00
*
PBS Control
15%
20%
25%
30%
35%
40%
45%
50%
55%
60%
65%
70%
AS Fractions

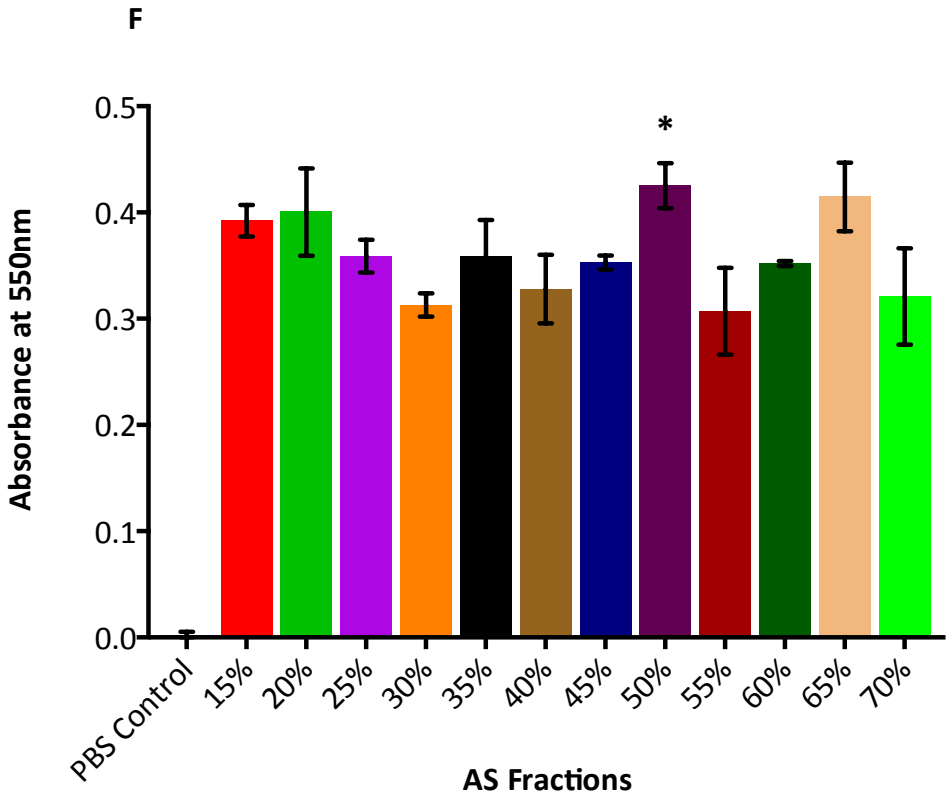


Figure 5.8 Ammonium sulfate precipitation fractions of P1097 BCM.

The protein precipitate fractions of *P. aeruginosa* (P1097) BCM were visualised using SDS-PAGE (**A** and **B**) and collagen zymography (**C** and **D**). Lanes 1 and 9 represent molecular weight marker. Controls include equine fibroblast-derived MMP-2 (lane 2) and DMEM culture medium (lane 3). Lanes 4-8 shows AS fractions 15, 20, 25, 30 and 35% and lanes 10-16 show fractions 40, 45, 50, 55, 60, 65 and 70% respectively. The remaining supernatant following AS precipitation is shown in lane 17. Subsequent protease assays of the AS precipitation fractions including the Azo-casein assay (**E**) and Azocoll assay (**F**) were performed. All experiments were repeated three times. Error bars represent standard error. Statistical analysis was performed using a Freidman non-parametric analysis of variants (ANOVA), with Dunn's multiple comparison test *P < 0.05. Abbreviations include biofilm-conditioned medium (BCM), Dulbecco's modified Eagles medium (DMEM), ammonium sulfate (AS) and phosphate buffered saline (PBS).

5.4 Discussion

Bacterial biofilms are a key area of clinical interest in the developed world and are being recognised as an important area of wound healing research by large associations such as the European wound management association (EWMA) (Kirketerp-Møller et al. 2011). Given the altered proteolytic environment of chronic wounds, it is important to consider whether this is, in part, due to the presence of a biofilm or the contribution of bacterial proteases themselves. Therefore this chapter aimed to utilise both *P. aeruginosa* and *S. aureus* obtained from human chronic wounds and assess whether these isolates displayed proteolytic activity in both planktonic and biofilm states.

In this study, there was a significant increase in proteases in the *P. aeruginosa* BCM when compared to PCM (see **Figures 5.4** and **5.5**), which may provide evidence that these extracellular proteases could play a role in biofilm formation. Indeed, there is existing evidence to suggest the involvement of bacterial intracellular proteases in biofilm formation. Marr and colleagues identified the gene encoding Lon protease to be induced in *P. aeruginosa* PA01 after exposure to sub-inhibitory concentrations of the antibiotic gentamicin. Furthermore, Lon protease mutants showed reduced bacterial swimming and twitching, two factors that are important in biofilm formation (Marr et al. 2007). Another more recent study tested the biofilm forming potential of *P. aeruginosa* that housed mutations in the protease-related proteins PfpI, ClpS, and ClpP and subsequently found up to a 70% reduction in biofilm formation when compared to the wild-type (Fernández et al. 2012). Research into the involvement of *P. aeruginosa* extracellular proteases in biofilm formation and EPS production has been documented. The over-expression of the *P. aeruginosa* extracellular protease elastase B (LasB) has been shown to significantly affect biofilm architecture, reduce alginate concentration, increase hydrophobicity and viscosity of EPS, and increase swimming, swarming and twitching motility of *P. aeruginosa* mucoid strains (Tielen et al. 2010). This evidence supports a role of bacterial extracellular proteases in biofilm formation. Indeed, the crystal violet assay showed varying levels of biofilm forming potential in *P. aeruginosa.* However comparisons between biofilm forming potential and proteolytic activity were not made in this

study. The reason for this is due to the difference in biofilm models used for the crystal violet assay (peg-lid method) and to obtain the BCM (grown on membrane filters), whereby these biofilms were grown on different surfaces with potentially different amounts of nutrient availability.

P. aeruginosa isolated from chronic wounds has been shown to produce proteases detected by zymography, of approximately 150kDa and 50kDa, which were identified as a multimeric form of *P. aeruginosa* elastase (33kDa) and alkaline protease (AprA) (50kDa) respectively (Schmidtchen et al. 2001). In addition, the zymography performed in the latter paper showed the presence of a faint proteolytic band around 64kDa; however the authors did not discuss this result. In my study, *P. aeruginosa* proteolytic bands of 52kDa and 62kDa were identified. It was initially thought that the 52kDa band may have been the metalloprotease AprA, however if this was the case, the proteolytic band should have been inhibited by 100mM EDTA, a known inhibitor of metalloproteases. Instead, the 100mM EDTA treatment resulted in no evidence of protease inhibition (see **Figure 5.7**). Therefore, at this stage of the research, the identification of the proteases that these clinical strains of *P. aeruginosa* are secreting remains unknown.

There are limitations to this study, firstly regarding the biofilm model used to obtain BCM. Though it is important that the biofilm is developed for at least 72 hours, it is in an artificial environment and the thickness of the biofilm *in vitro* is probably not reflective of a biofilm that resides in the human tissue of a chronic wound. In addition to this, the proteolytic activity that has been documented in this study may differ in an *in vivo* setting, where factors such as nutrient availability and host-derived growth factors and cytokines may have an effect. Furthermore, in order to normalise the protease results between PCM and BCM, the bacterial cell numbers for the preparation of PCM were diluted to similar bacterial cell numbers of the 72-hour biofilms. However this method of normalisation has limitations in that it is an approximate, as each bacterial strain has slightly different growth rates, not to mention the growth rate of a biofilm slows as the biofilm matures (Costerton et al.

1999). In addition, the experimental environment in the production of PCM and BCM differ. The bacteria for PCM is grown for 24 hours in nutrient-rich conditions, however the biofilms are grown for 72 hours on the surface of membrane filter discs with potentially limited nutrients from the agar beneath due to the 0.2µm pore surface of the filter. However, it has been documented that nutrient depletion encourages biofilm formation as a mechanism to survive hostile environments and that the introduction of nutrient sources causes a reduction in biofilm biomass through dispersal (O'Toole et al. 2000, Sauer et al. 2004).

5.4.1 Future directions

In this study, I have demonstrated that *P. aeruginosa* in biofilm form produce high levels of proteases. This led to the discovery of two proteolytic bands at 52kDa and 62kDa produced by some of the isolates. Although uncertainty still surrounds the identification of these bands, it would be interesting to determine the effect of these partially purified bacterial proteases on wound closure, in order to determine whether they may play a role in reduced wound closure in infected chronic wounds. To do so, initial studies could investigate the effect PCM, BCM and the partially purified bacterial proteases on fibroblast and keratinocyte wound closure in an *in vitro* scratch wound model. The presence of MRSA PCM and BCM on human dermal fibroblasts has been shown to reduce cellular migration and viability *in vitro*. Furthermore, differential effects in MMP expression in human dermal fibroblasts was recorded, with MRSA PCM causing an increase in MMP-1 and MMP-3 production compared to MRSA BCM (Kirker et al. 2012). Thus, as the bacterial isolates used in the present study are of chronic wound origin and not a typical laboratory strain, it would be interesting to investigate the effect of both *S. aureus* and *P. aeruginosa* clinical isolates, in planktonic and biofilm form on the expression and production of host proteases in a similar model. Furthermore, it would be intriguing to determine whether protease expression and production is altered in the presence of the partially purified *P. aeruginosa*-derived proteases. This type of study could

potentially reveal whether bacterial-derived proteases play a role in imbalances in MMP production observed in the chronic wound environment.

5.4.2 Key points

In summary, *P. aeruginosa* isolated from human chronic wounds displayed high levels of proteolytic activity when grown in biofilm form. However planktonic growth of these *P. aeruginosa* isolates revealed little or no proteolytic activity. Using zymographic analysis, two proteolytic bands were evident in *P. aeruginosa* BCM at 52kDa and 62kDa, which the major broad-spectrum protease inhibitors failed to inhibit. The identification of these proteases remains unknown.

S. aureus isolated from human chronic wounds showed little evidence of proteolytic activity in both planktonic and biofilm forms.

CHAPTER 6:

Results IV: The Effect of *S. aureus* and *P. aeruginosa* Planktonic- and Biofilm-Conditioned Medium on Human Dermal Fibroblast and Human Epidermal Keratinocyte Wound Closure and Protease Secretion *In Vitro*

6 Results IV: The Effect of *S. aureus* and *P. aeruginosa* Planktonic- and Biofilm-Conditioned Medium on Human Dermal Fibroblast and Human Epidermal Keratinocyte Wound Closure and Protease Secretion *In Vitro*

6.1 Introduction

A chronic wound can be defined as a wound that is slow to heal and that is halted in the inflammatory phase of the wound healing process (Velnar et al. 2009). Chronic wounds such as pressure ulcers, venous leg ulcers and diabetic foot ulcers, present with an increased infiltration of neutrophils, increased levels of pro-inflammatory cytokines and an exaggerated proteolytic environment. There are known host factors that contribute to the development of a chronic wound, such as ischemia, diabetes and abnormal white blood cell function (Guo and DiPietro 2010). However it has been proposed that it is not these factors that prevent a wound from healing, rather the compromised immune response to invading bacteria, which are then able to form a biofilm, resulting in persistent inflammation (Wolcott et al. 2008). Clinical studies and *in vivo* laboratory research have shown that the presence of a biofilm may be detrimental to the wound healing process. For example, clinical studies by Hurlow and Bowler described the presence of a slimy film, distinct from that of slough, in wounds showing delayed wound healing (Hurlow and Bowler 2009). This theory is strengthened by work investigating the use of photodynamic therapy (PDT), which uses irradiation coupled with a photoactive dye resulting in microbial cell death, which resulted in improved wound healing. Morley and colleagues showed a significant reduction in bacterial load after PDT treatment in patients with chronic leg ulcers when compared to the placebo controls in a phase IIa, randomised, blinded and single treatment study (Morley et al. 2013). Results showed complete wound closure in 50% of patients that underwent PDT when compared to 12% of patients on placebo. Animal studies such as that of Gurjala and co-workers, have shown that the presence of *S. aureus* biofilms in wounded New Zealand rabbit ears, results in sustained low grade inflammation, decreased granulation tissue formation and reduced re-epithelialisation (Gurjala et al. 2011).

As investigated in the previous chapter, the presence of bacterial biofilms in chronic wounds could potentially contribute to the exaggerated proteolytic environment that has been documented in chronic wounds, given the high extracellular protease production from chronic wound isolates such as *P. aeruginosa* (see Chapter 5). Whether the presence of bacterial biofilms in a wound promotes the excessive release of proteases from host cells, has yet to be determined. A study by Twining and colleagues assessed the effect of *P. aeruginosa* extracellular proteases, elastase and alkaline protease (AprA) on the secretion of proteases from rabbit corneas (Twining et al. 1993). The results of the latter study showed that *P. aeruginosa*-derived extracellular proteases could activate corneal proteases, with *P. aeruginosa* elastase cleaving a 92kDa corneal gelatinase into an active 77kDa form. Furthermore the authors reported release of a 72kDa gelatinase and 97kDa caseinase in presence of the *P. aeruginosa* virulence factor exotoxin A. This research highlighted the potential interaction between host tissue and bacteria in the release and activation of active proteases that can lead to tissue destruction. The effect of bacterial biofilms on host-protease expression in the context of chronic wounds has not been fully addressed. Cell types such as dermal fibroblasts play critical roles in all aspects of wound repair such as wound contraction and extracellular matrix (ECM) turnover, through the production of ECM components including collagen-I and the release of matrix metalloproteases (MMPs) (Bainbridge 2013). Furthermore, increases in the expression of MMP-2 and MMP-3 in fibroblasts from human diabetic foot ulcers has been documented (Wall et al. 2003). Keratinocytes, the major cell type in the epidermis, play an important role in maintaining barrier function against invading microorganisms. Failure to re-epithelialise is a characteristic that is commonplace in chronic wounds.

It was therefore the aim of this chapter to assess the effect of human chronic wound-derived *S. aureus* and *P. aeruginosa* planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM) on the wound closure of adult human dermal fibroblasts (HDFs) and adult human epidermal keratinocytes (HEKs). The intracellular expression and extracellular secretion of host-derived proteases was investigated to determine whether these cells altered the secretion of proteases in response to

bacterial-conditioned medium. This chapter also investigated the effect of the *P. aeruginosa*-derived partially purified protease, outlined in chapter 5 of this thesis, on HDFs and HEKs wound closure. Finally this study aimed to assess the effect of bacterial-conditioned medium on host protease expression in a more physiologically relevant model, which required the development of a 3-dimensional keratinocyte-fibroblast co-culture *in vitro*. Based on the findings in previous chapters, I hypothesised that the treatment of HDFs and HEKs with *P. aeruginosa* PCM and BCM, would significantly reduce wound closure *in vitro*. Furthermore, I hypothesised that *P. aeruginosa*-derived partially purified proteases would also reduce HDFs and HEKs wound closure.

6.2 Methods

For a detailed description of the methods used in this chapter, the reader is directed to chapter 2 of this thesis (2.3 Microbiology, 2.7 Gel Electrophoresis, 2.8 Ammonium Sulfate Precipitation and Partial Purification of Proteins, 2.9 BCA Assay and Sample Protein Quantification, 2.10 Cell Culture, 2.13 Scratch Wound Assay, 2.14 Cell Viability Assay, 2.15 3D Keratinocyte-Fibroblast Co-culture Model, 2.16 Quantitative RT-PCR, 2.17 Histology and Immunohistochemistry and 2.18 Statistical Analysis).

6.3 Results

6.3.1 Human dermal fibroblast (HDF) scratch closure and cellular viability in response to P. aeruginosa and S. aureus planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM)

In order to determine whether chronic wound-derived *P. aeruginosa* and *S. aureus* had any effect on the wound closure of human dermal fibroblasts (HDFs), HDFs were treated with either PCM or BCM of each bacterial isolate in an *in vitro* scratch wound assay. Prior to starting the experiment, PCM and BCM were obtained as the methods describe in Chapter 2, and were subsequently diluted 1:1 with DMEM (without

antibiotics) before adding to the HDF scratches. Images of the scratch wounds taken at 0, 24 and 48 hours showed that *P. aeruginosa* BCM greatly reduced scratch closure when compared to all other treatment groups and in some cases caused cell death (see **Figure 6.1**). Neither *S. aureus* PCM or BCM appeared to affect closure. Following ImageJ analysis of the scratch wounds, it was found that within 24 hours, control scratches (treated with DMEM complete) were 59.8% ± 13.8% closed, while *P. aeruginosa* PCM and BCM were 49.1% ± 16.8% and 26.6% ± 17.2% closed respectively, with *P. aeruginosa* BCM-treated scratches showing a significant reduction in closure when compared to control and *P. aeruginosa* PCM ($P < 0.001$) (see **Figure 6.2**). After 48 hours, control scratches were 93.7% ± 5.5% closed, which was comparable to scratches treated with *S. aureus* PCM that showed 95.0% ± 6.9% closure. In contrast, scratches treated with *P. aeruginosa* BCM showed significantly reduced closure (40.1% ± 20.4%) at 48 hours ($P < 0.0001$) when compared with control, *P. aeruginosa* PCM and *S. aureus* BCM. A reduction in closure was seen after 48 hours for scratches treated with *P. aeruginosa* PCM (79.4% ± 21.3%), which was significant when compared to *S. aureus* PCM ($P < 0.01$).

Given that percentage scratch closure was significantly reduced by *P. aeruginosa* BCM at 24 and 48 hours (see **Figure 6.1** and **Figure 6.2**), the PrestoBlue™ cell viability reagent was employed to test whether there was a reduction in the viability of these cells in response to the bacterial-conditioned medium (see **Figure 6.3**). After 4 hours treatment with bacterial-conditioned medium, no significant changes in viability between control scratches and the treatment groups were recorded. Similarly, after 24 hours, HDFs treated with bacterial-conditioned medium did not show any significant reduction in viability when compared with the control, although differences between treatment groups were recorded. There was a significant reduction in the viability of cells treated with *S. aureus* BCM when compared with *S. aureus* PCM and *P. aeruginosa* BCM-treated cells showed a significant reduction in viability when compared to *S. aureus* BCM ($P < 0.0001$). After 48 hours of treatment the viability of HDFs treated with *S. aureus* and *P. aeruginosa* BCM was significantly lower than that of the control ($P < 0.01$ and $P < 0.0001$ respectively), with *P. aeruginosa* BCM treatment causing the greatest reduction in cell viability. The

reduced viability seen in *P. aeruginosa* BCM-treated cells was significantly lower than other treatment groups including *P. aeruginosa* PCM and *S. aureus* BCM ($P < 0.0001$). Furthermore *S. aureus* BCM treated cells showed a greater reduction in viability than *S. aureus* PCM ($P < 0.0001$).

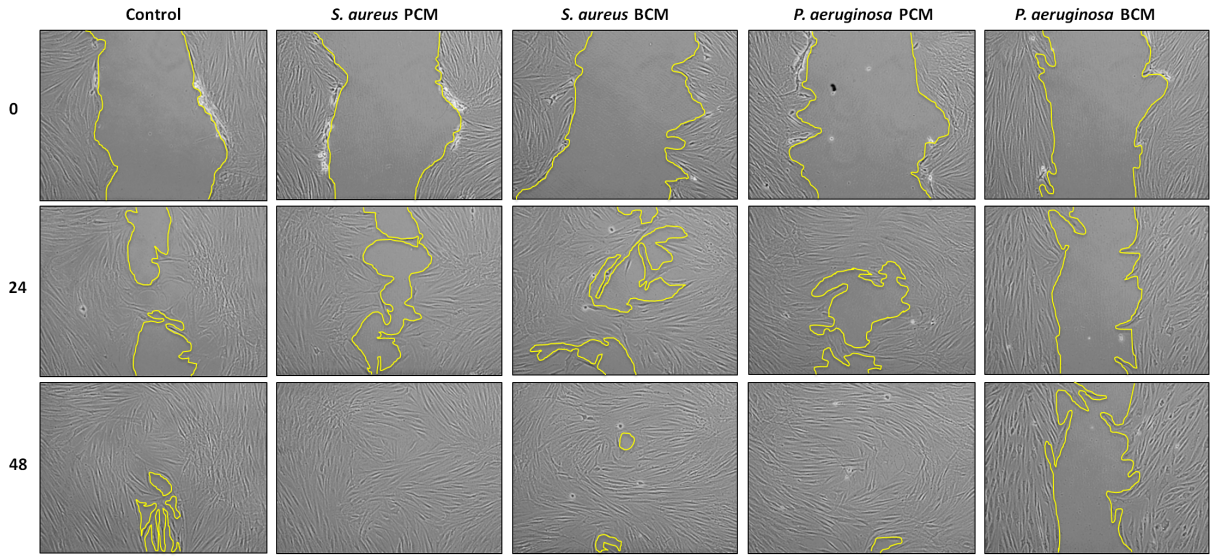


Figure 6.1 Representative images of scratch wounds after treatment with human chronic wound-derived *S. aureus* and *P. aeruginosa* planktonic- and biofilm-conditioned medium.

Monolayer cultures of adult human dermal fibroblasts (HDFs) were scratched before adding either control medium or bacterial-conditioned medium. Images of the scratch area were then taken at 0, 24 and 48 hours, post-treatment, using the Nikon Eclipse microscope. Yellow lines outline the scratch wound area. Abbreviations include planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

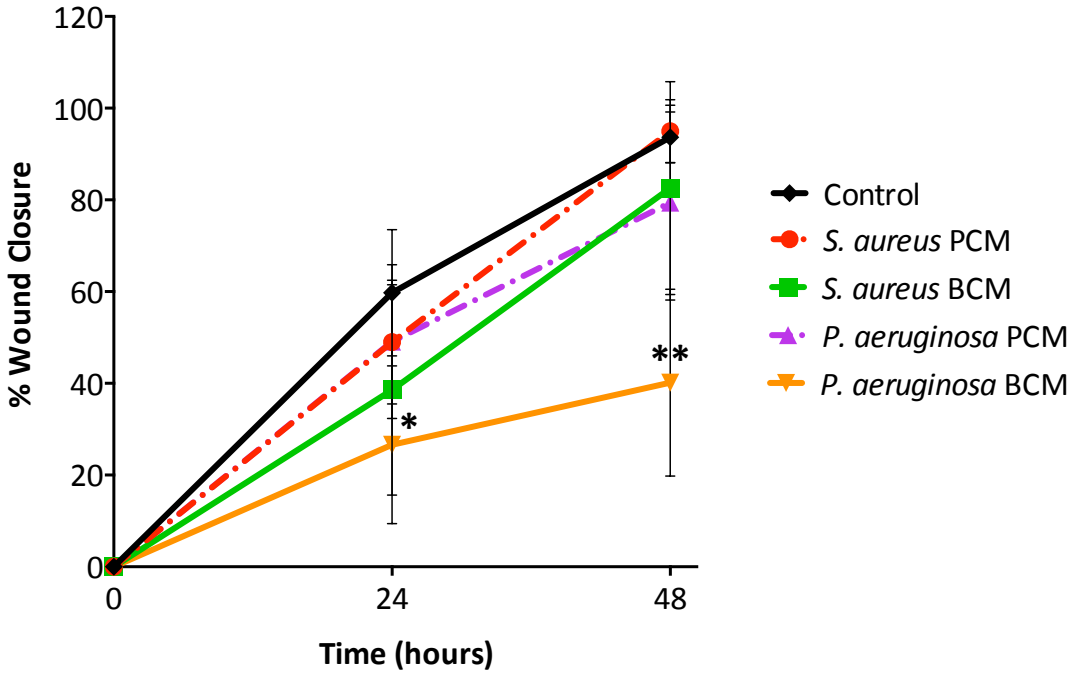


Figure 6.2 Percentage scratch wound closure of adult human dermal fibroblasts (HDFs) *in vitro*.

Results are shown for HDFs treated with either *S. aureus* or *P. aeruginosa* planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM). Results represent the mean values ± standard deviation (biological replicates: *S. aureus* n=19 and *P. aeruginosa* n=15, technical replicates n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.001 and **P <0.0001 when compared with the control group.

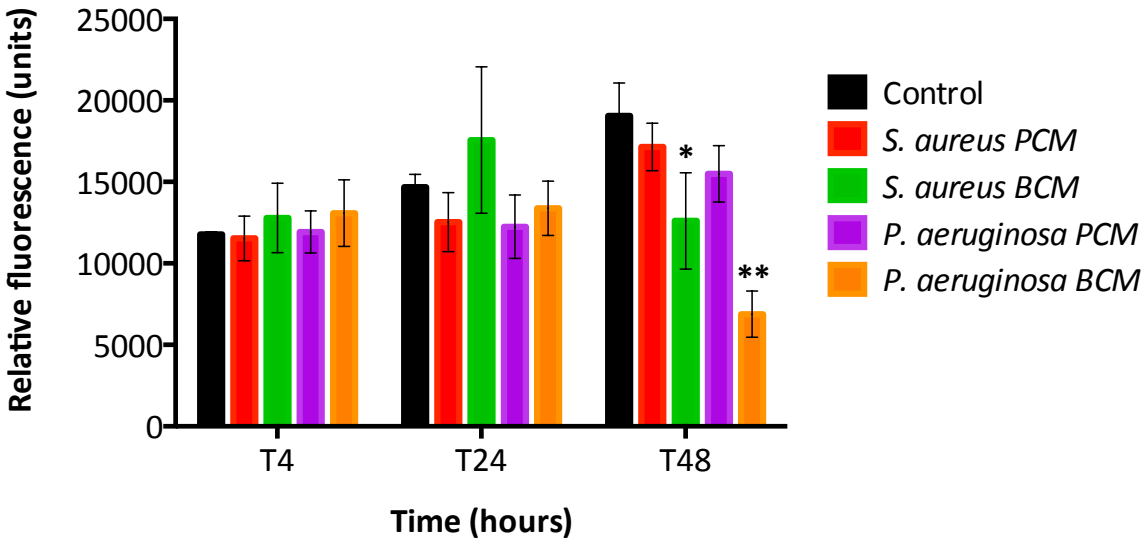


Figure 6.3 Cell viability of adult human dermal fibroblasts (HDFs) using PrestoBlue™ *in vitro*.

Results are shown as relative fluorescence values whereby HDFs were exposed to *S. aureus* and *P. aeruginosa* planktonic-conditoned medium (PCM) and biofilm-conditioned medium (BCM) over 48 hours. Results represent the mean values ± standard deviation (biological replicates: *S. aureus* n=19 and *P. aeruginosa* n=15, technical replicates n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.01 and **P <0.0001 when compared with the control group.

6.3.2 Secretion and expression of adult human dermal fibroblast (HDFs) matrix metalloproteases (MMPs) in response to P. aeruginosa and S. aureus planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM)

To establish whether the presence of *S. aureus* and *P. aeruginosa* bacterial-conditioned medium had any affect on the secretion of active host-derived

proteases, gelatin zymography was used to assess the supernatant from the *in vitro* scratch assay at 24 and 48 hours (see **Figure 6.4**). Control supernatants, including DMEM culture medium alone, untreated no-scratch control (NSC) and untreated scratch control, all contained two proteases of approximately 60kDa and 80kDa (see **Figure 6.4, A**). When evaluating the supernatants from scratches treated with *P. aeruginosa* PCM, zymography showed an increase in the intensity of the 60kDa band at 24 hours and again at 48 hours when compared to the *P. aeruginosa* PCM alone. Furthermore, the presence of the 80kDa protease remained unchanged in the *P. aeruginosa* PCM-treated cell supernatants. Supernatants treated with *P. aeruginosa* BCM showed the presence of the bacterial-derived 62kDa protease, which was identified in the previous chapter. Interestingly, at both 24 and 48 hours of treatment with the *P. aeruginosa* BCM, the appearance of an additional protease band of approximately 56kDa was noted (see red arrow, **Figure 6.4, A**). Additionally the 80kDa protease was not observed in either *P. aeruginosa* BCM alone, or in the 24- and 48-hour scratch supernatants (see black arrow, **Figure 6.4**, **A**). To determine whether the additional protease, seen in the BCM-treated cell supernatants, belonged to the metalloprotease group, the zymograms were inhibited with 100mM EDTA (see **Figure 6.4, B**). The results showed a complete inhibition of the 56kDa protease and the proteases identified in the control and PCM supernatants, and was therefore undetectable on the zymograms. However, as shown in Chapter 5 of this thesis, the *P. aeruginosa*-derived 62kDa protease in the BCM supernatants remained uninhibited by the EDTA treatment and was therefore still observed on the zymograms. The treatment of HDFs with *S. aureus* PCM and BCM caused a change in the intensity of the 60kDa band after 48 hours in the scratch assay (see **Figure 6.5**). Furthermore, the 80kDa was faint but present in all samples. All protease bands detected by gelatin zymography were completely inhibited by 100mM EDTA (data not shown). The presence of any further proteases within the supernatant of *S. aureus* PCM- and BCM-treated HDFs were not detected.

The expression of MMPs in HDFs at a transcriptional level was assessed using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to determine whether any significant changes in MMP mRNA expression occurred after 48 hours

treatment with bacterial-conditioned medium in the scratch assay (see **Figure 6.6**). More specifically, the collagenases MMP-1 and MMP-13 and gelatinases MMP-2 and MMP-9 were investigated. Furthermore, expression of the major substrate for the collagenases (and also the gelatinases), collagen-I, was tested. The validity of the expression data for MMP-9 and MMP-13 were considered unreliable given high delta Ct values of approximately 30 (data not shown) and therefore were not presented in this chapter. The reason for unreliable expression data could be due to a lack of expression of MMP-9 and MMP-13 in HDFs. The expression of collagen-I in HDFs was significantly reduced in cells treated with *P. aeruginosa* BCM when compared to the untreated scratch control ($P < 0.05$). When evaluating the MMP-1 expression data, a significantly higher expression of MMP-1 in cells treated with *P. aeruginosa* PCM when compared to *S. aureus* PCM ($P < 0.01$) was found. MMP-2 expression in all treatment groups remained relatively constant with the untreated scratch control. In all expression data, there were large error bars indicating variability in the results obtained between each biological replicate in their respective treatment groups.

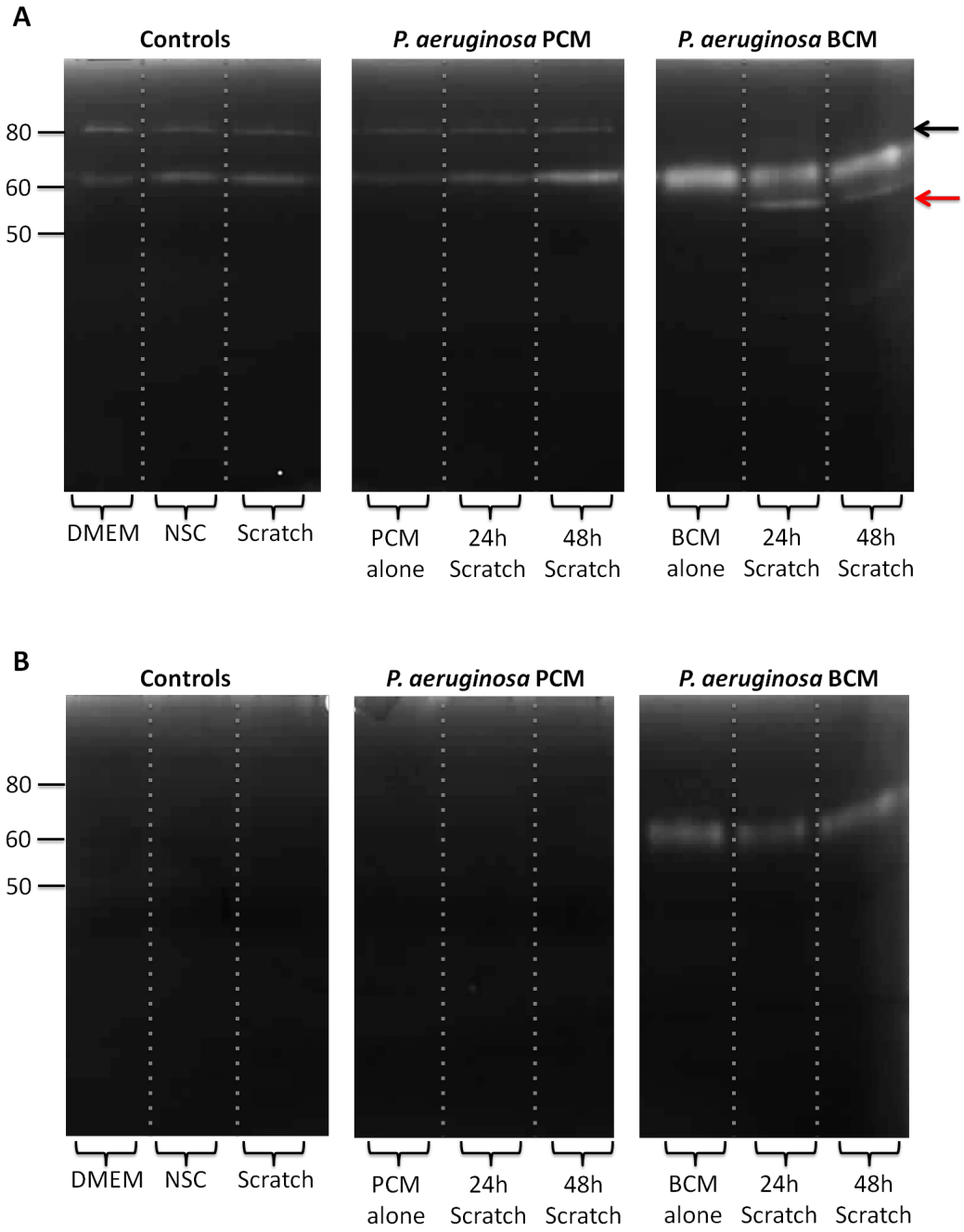


Figure 6.4 Representative zymography of supernatants from the HDFs scratch wound assay following *P. aeruginosa* PCM and BCM treatment.

Supernatants from *P. aeruginosa* PCM- and BCM-treated cells after 24 and 48 hours were run on gelatin zymograms, which were either untreated (**A**) or inhibited for metalloproteases using 100mM EDTA (**B**). Controls include DMEM culture medium alone, untreated no-scratch control and untreated scratch control. Red and black arrows represent points of interest, discussed in the text. Gelatin zymography of all supernatants from *P. aeruginosa* PCM- and BCM-treated cells were run and repeated twice (technical replicates, n=2). This zymogram is representative of HDFs treated with *P. aeruginosa* P1126 clinical isolate. The molecular weight is in kilodaltons (kDa).

Abbreviations include Dulbecco's modified Eagles medium (DMEM), no-scratch control (NSC), planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

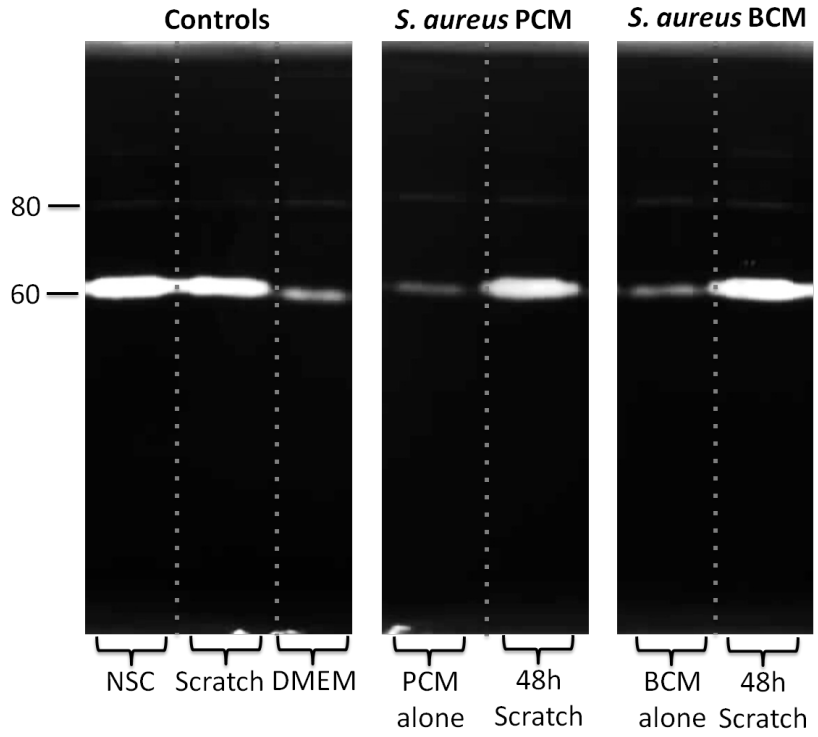
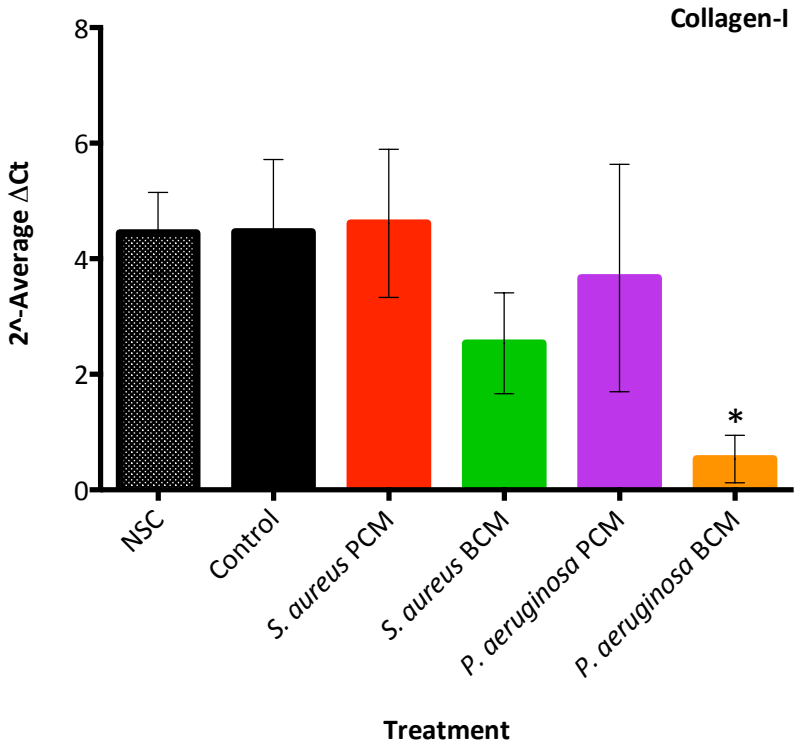


Figure 6.5 Representative zymography of supernatants from HDF scratch wound assay following *S. aureus* PCM and BCM treatment.

Supernatants from *S. aureus* PCM- and BCM-treated cells after 48 hours were run on gelatin zymograms. Controls include DMEM culture medium alone, untreated no-scratch control and untreated scratch control. Gelatin zymography of all supernatants from *S. aureus* PCM- and BCM-treated cells were run and repeated twice (technical replicates, n=2). The molecular weight is in kilodaltons (kDa). Abbreviations include Dulbecco's modified Eagles medium (DMEM), no-scratch control (NSC), planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).


Collagen-I
2^-Average ΔCt
8
6
4
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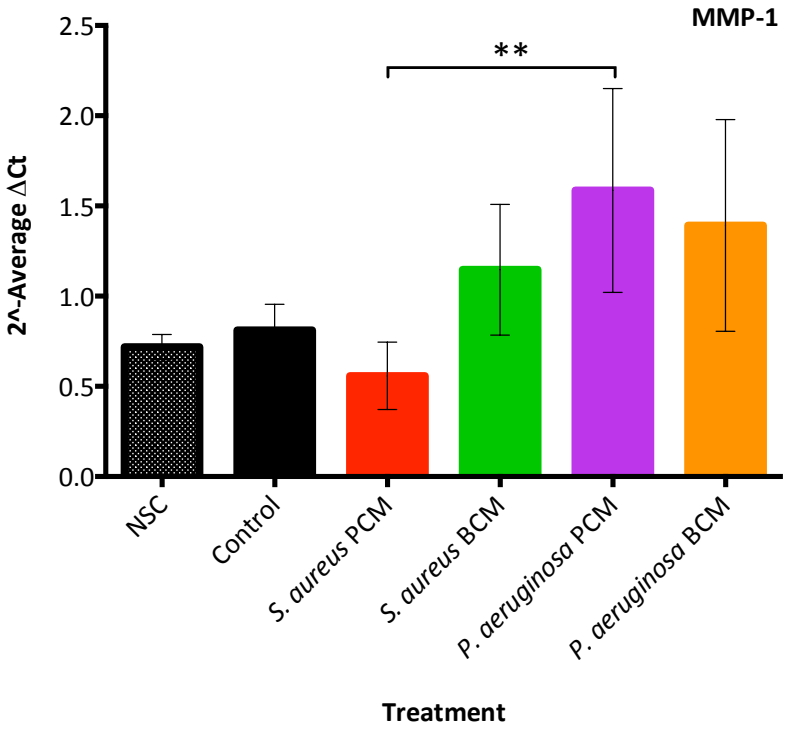
*
NSC
Control
S. aureus PCM
S. aureus BCM
P. aeruginosa PCM
P. aeruginosa BCM
Treatment


MMP-1
2^-Average ΔCt
2.5
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NSC
Control
S. aureus PCM
S. aureus BCM
P. aeruginosa PCM
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Treatment

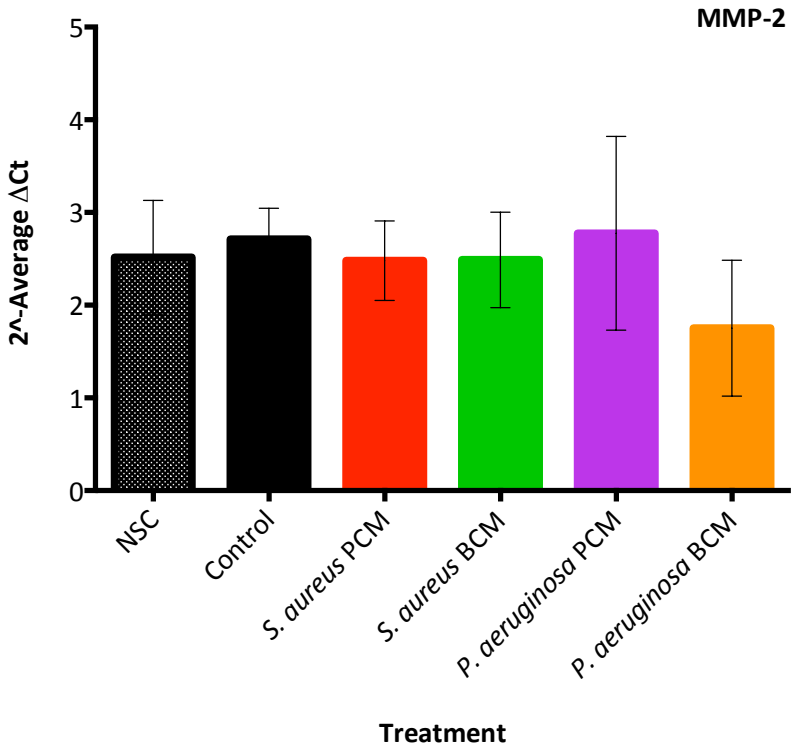

Figure 6.6 The expression of MMPs and collagen-I using quantitative RT-PCR.

Results represent the mean values ± standard deviation (biological replicates for each species n=6, technical replicates n=2). Statistical analysis was performed using the non-parametric Kruskal-Wallis with Dunn's multiple comparisons test. Statistical significance is indicated as *P <0.05 and **P <0.01. Abbreviations include no-scratch control (NSC), matrix metalloprotease (MMP), planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM).

6.3.3 The effect of P. aeruginosa and S. aureus planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM) on adult human epidermal keratinocytes (HEKs)

Scratch assays using HEKs were performed in the same manner as that of the HDFs scratch assays, to determine whether *S. aureus* and *P. aeruginosa* PCM and BCM had similar effects on wound closure and cell viability. Images of scratch closure were

taken at 0, 24 and 48 hours and were analysed using ImageJ software. After 24 hours, the untreated scratch control cells showed 25.4% ± 12.1% closure, with cells treated with *S. aureus* PCM and BCM showing mixed outcomes between biological replicates with 49.4% ± 46.4% and 11.1% ± 45.3% closure respectively (see **Figure 6.7**). Cells treated with *P. aeruginosa* PCM and BCM after 24 hours showed -15.0% ± 132.2% and -36.7% ± 56.9% closure. After 48 hours the untreated control cells had reached 83.0% ± 7.6% closure, however cells treated with *S. aureus* PCM and BCM continued to have profound effects on closure with percentage values of 29.3% ± 107.7% and -17.4% ± 85.3%. Similarly, most scratches treated with *P. aeruginosa* PCM and BCM resulted in cell detachment, giving percentage closure values of 1.9% ± 99.0% and -95.8% ± 53.6%. There were huge variability in these results; however, statistical analysis revealed that *P. aeruginosa* BCM significantly reduced wound closure when compared to the untreated control ($P < 0.01$) and cells treated with *P. aeruginosa* PCM ($P < 0.05$).

It was clear from scratch wound analysis, that all bacterial-conditioned medium had a great effect on not only HEKs scratch closure, but also the viability of the cells, with the majority of cells treated with *P. aeruginosa*-conditioned medium showing cell death. Therefore the cell viability of untreated HEKs and HEKs treated with the bacterial-conditioned medium were assessed. Results showed a large amount of variability in the viability of HEKs in all treatment groups after 24 and 48 hours (demonstrated by standard deviation error bars), which reflected the variability seen in the scratch closure results (see **Figure 6.8**). At 4 hours post-treatment there were no differences in cells treated with bacterial-conditioned medium when compared to the untreated control. After 24 hours, there were significant reductions in keratinocyte viability upon treatment with *S. aureus* BCM ($P < 0.05$) and *P. aeruginosa* BCM ($P < 0.01$) when compared to the untreated control. After 48 hours, only HEKs treated with *P. aeruginosa* BCM showed a significantly lower viability when compared to the untreated control ($P < 0.05$).

Due to the obvious cell death observed in many of the treatment groups in the HEKs scratch assays, zymography of the scratch assay supernatants and quantitative RT-PCR was not performed.

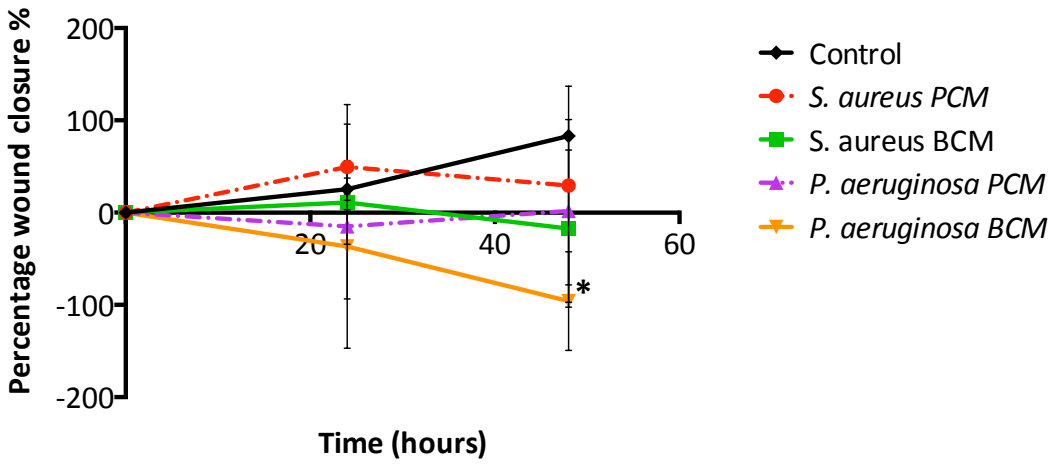

Figure 6.7 Percentage scratch wound closure of adult human epidermal keratinocytes (HEKs) *in vitro*.

Results are shown for HEKs treated with either *S. aureus* or *P. aeruginosa* planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM). Results represent the mean values ± standard deviation (biological replicates: *S. aureus* n=19 and *P. aeruginosa* n=15, technical replicates n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.01 when compared with the control group.

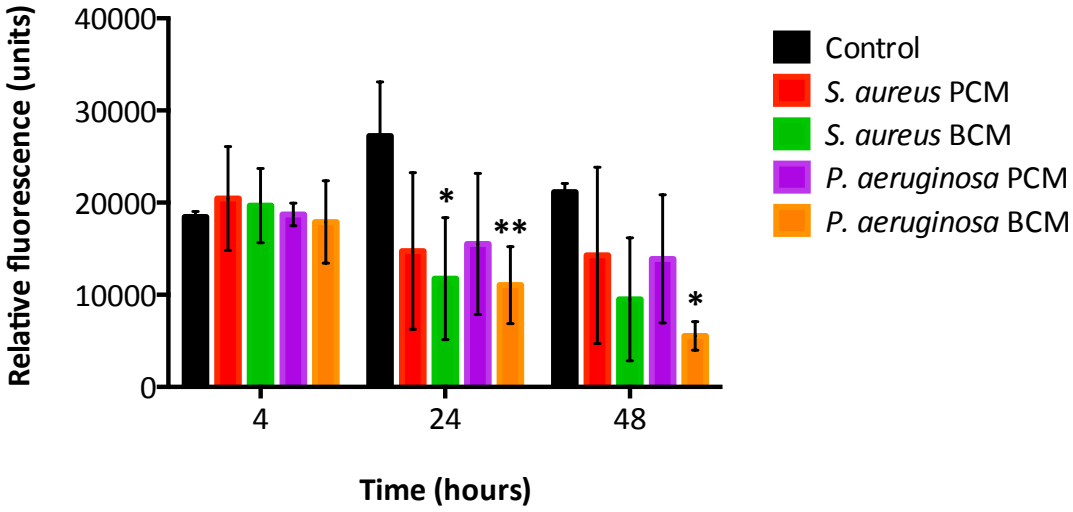

Figure 6.8 Adult human epidermal keratinocyte (HEK) viability following bacterial-conditioned medium treatment using PrestoBlue™.

Results are shown as relative fluorescence values whereby HEKs were exposed to *S. aureus* and *P. aeruginosa* planktonic-conditoned medium (PCM) and biofilm-conditioned medium (BCM) over 48 hours. Results represent the mean values ± standard deviation (biological replicates: *S. aureus* n=19 and *P. aeruginosa* n=15, technical replicates n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.05 and **P <0.01 when compared with the untreated control group.

6.3.4 The scratch closure and cellular viability of adult human dermal fibroblasts (HDFs) and adult human epidermal keratinocytes (HEKs) in response to P. aeruginosa-derived, partially purified proteases

P. aeruginosa isolated from human chronic wounds has shown high levels of extracellular protease activity and secretes proteases of approximately 52kDa and 62kDa (presented in the previous chapter). These proteases were partially purified using an ammonium sulfate precipitation and dialysis protocol, and were used to

determine their effect on HDFs and HEKs scratch closure and cell viability. Both 50 μg/ml and 100 μg/ml of the partially purified protease (isolated from human chronic wound-originating *P. aeruginosa* P1097, P1140, P1145 BCM) had no significant effect on scratch closure in HDFs (see **Figure 6.9**). The cellular viability of HDFs was tested and there was a significant reduction in viability after 48 hours exposure to 100 μg/ml of the partially purified proteases ($P < 0.01$) (see **Figure 6.10**). Given that both PCM and BCM of both *S. aureus* and *P. aeruginosa* had a greater effect on keratinocyte scratch wound closure than HDFs wound closure, the effect of the partially purified proteases on keratinocyte scratch closure was tested. After 24 hours, a significant reduction in closure of -120.2% ± 103.4 was seen after treatment with 100 μg/ml of the partially purified proteases ($P < 0.05$) (see **Figure 6.11**). After 48 hours both 50 μg/ml and 100 μg/ml of the partially purified proteases caused a statistically significant reduction in closure of -112.9% ± 119.7% and -151% ± 57.3% respectively ($P < 0.01$). Treatment with the partially purified proteases also had a greater effect on cell viability in the keratinocytes than fibroblasts. After 24 hours, there was a significant reduction in keratinocyte viability with 50 μg/ml and 100 μg/ml of the partially purified protease when compared to the untreated control ($P < 0.0001$). After 48 hours, keratinocyte viability remained significantly lower than the untreated control ($P < 0.001$) (see **Figure 6.12**).

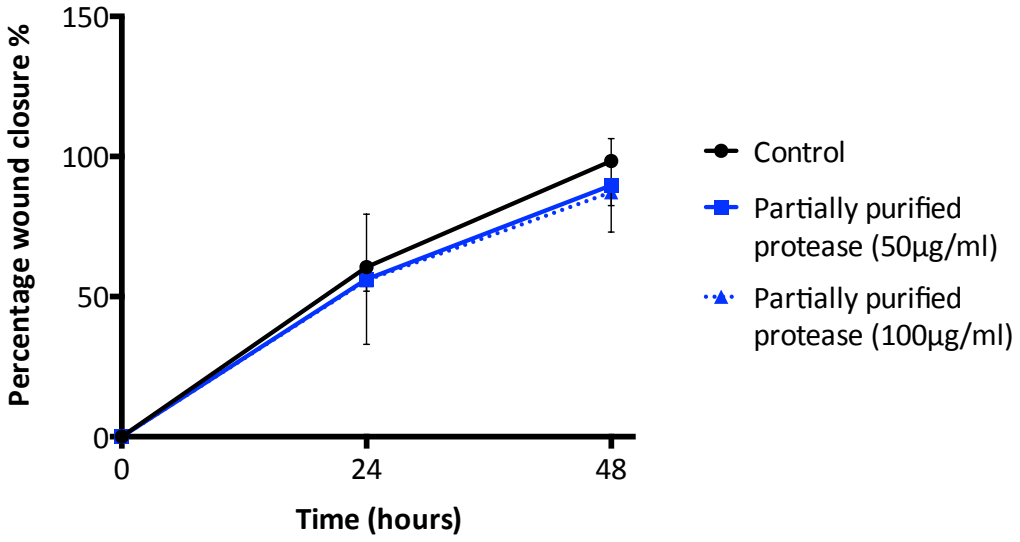

Figure 6.9 Percentage scratch wound closure of adult human dermal fibroblasts (HDFs) after treatment with *P. aeruginosa*-derived partially purified proteases.

Results represent mean values ± standard deviation (biological replicates: n=3, technical replicates n=2). There was no statistically significant difference in the percentage scratch wound closure between HDFs treated with the partially purified proteases and HDFs treated with control (DMEM complete) medium alone.

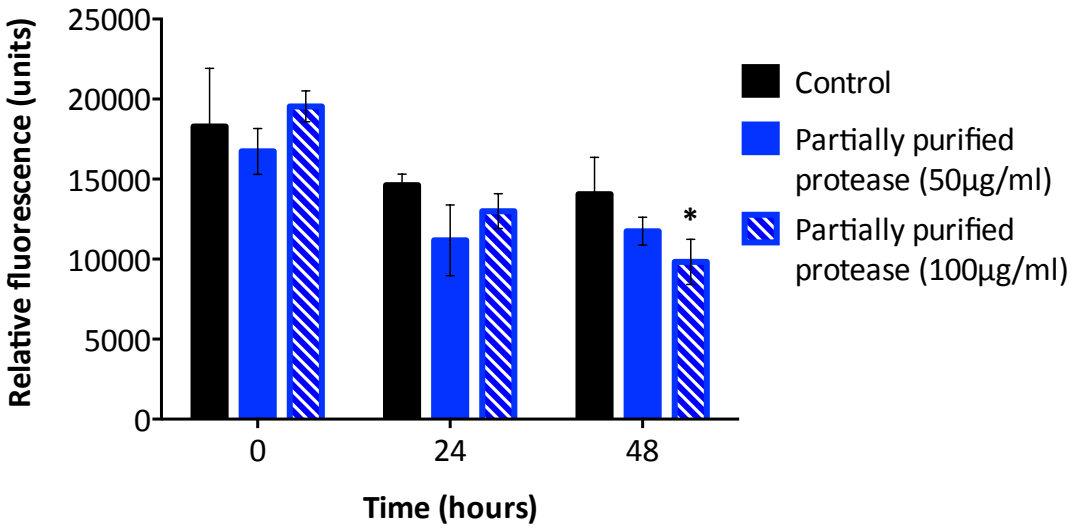

Figure 6.10 Cell viability of adult human dermal fibroblasts (HDFs) after treatment with *P. aeruginosa*-derived partially purified proteases, using PrestonBlue™.

Results show two treatment groups, whereby HDFs were treated with 50 µg/ml and 100 µg/ml *P. aeruginosa*-derived protease and the control group whereby HDFs were treated with DMEM complete. Results represent the mean values ± standard deviation (biological replicates n=3, technical replicates n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P < 0.01 when compared with the control group.

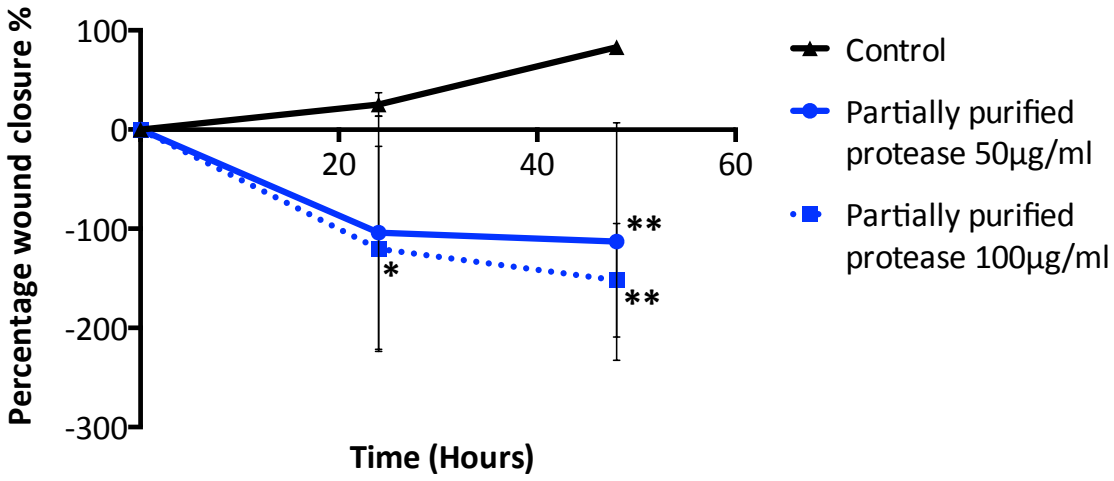

Figure 6.11 Percentage scratch wound closure of adult human epidermal keratinocytes (HEKs) after treatment with *P. aeruginosa*-derived partially purified proteases.

Results represent mean values ± standard deviation (biological replicates: n=3, technical replicates: n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P <0.05 and **P <0.01 when compared with the control group.

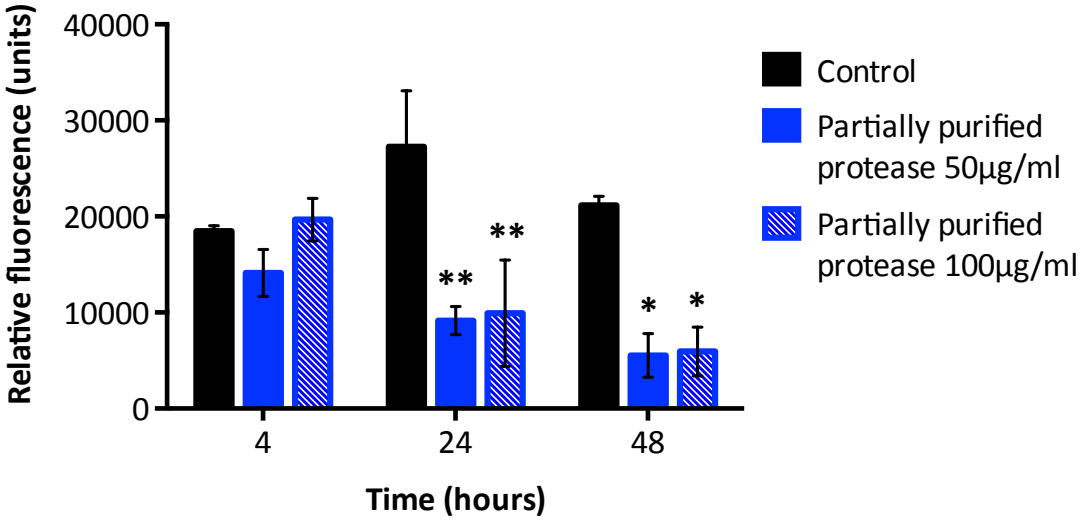

Figure 6.12 Cell viability of adult human epidermal keratinocytes (HEKs) after treatment with *P. aeruginosa*-derived partially purified proteases, using PrestoBlue™.

Results show two treatment groups, where HEKs were treated with 50 µg/ml and 100 µg/ml *P. aeruginosa*-derived protease. Results represent the mean values ± standard deviation (biological replicates n=3, technical replicates n=2). Statistical analysis was performed using two-way analysis of variants (ANOVA) with Tukey's multiple comparisons test. Statistical significance is indicated as *P < 0.001 and **P <0.0001 when compared with the control group.

6.3.5 The development of a 3-D keratinocyte and fibroblast co-culture model

To assess the effect of *P. aeruginosa* and *S. aureus* PCM and BCM on wound closure in a more physiological model than a monolayer scratch wound, a co-culture of HEKs and HDFs within a 3-Dimensional fibrin matrix was developed, based on the work of (Holland et al. 2008). In the first attempt at the model, the keratinocytes adhered in a patchy manner with the majority of cells aggregating in the centre of the dermal fibroblast layer surface. In addition, it was observed that a large number of the HEKs seeded on the dermal layer failed to adhere. Nevertheless, the co-culture model

continued to be cultured before fixing and assessing the preliminary model histologically. Haematoxylin and eosin (H&E) staining revealed a thick keratinocyte layer that was dense in cell nuclei (see **Figure 6.13, A**). The dermal layer was faintly stained, with few identifiable fibroblasts (see **Figure 6.13, B**). Despite this, a distinct layer between the adhered keratinocytes and the fibroblast dermal layer was observed, which was proposed to be the formation of the basement membrane (see **Figure 6.13, C**). Unfortunately, it was not possible to develop this model further planned attempts at this model due to difficulties with the culturing and viability of HEKs on the dermal layer construct.

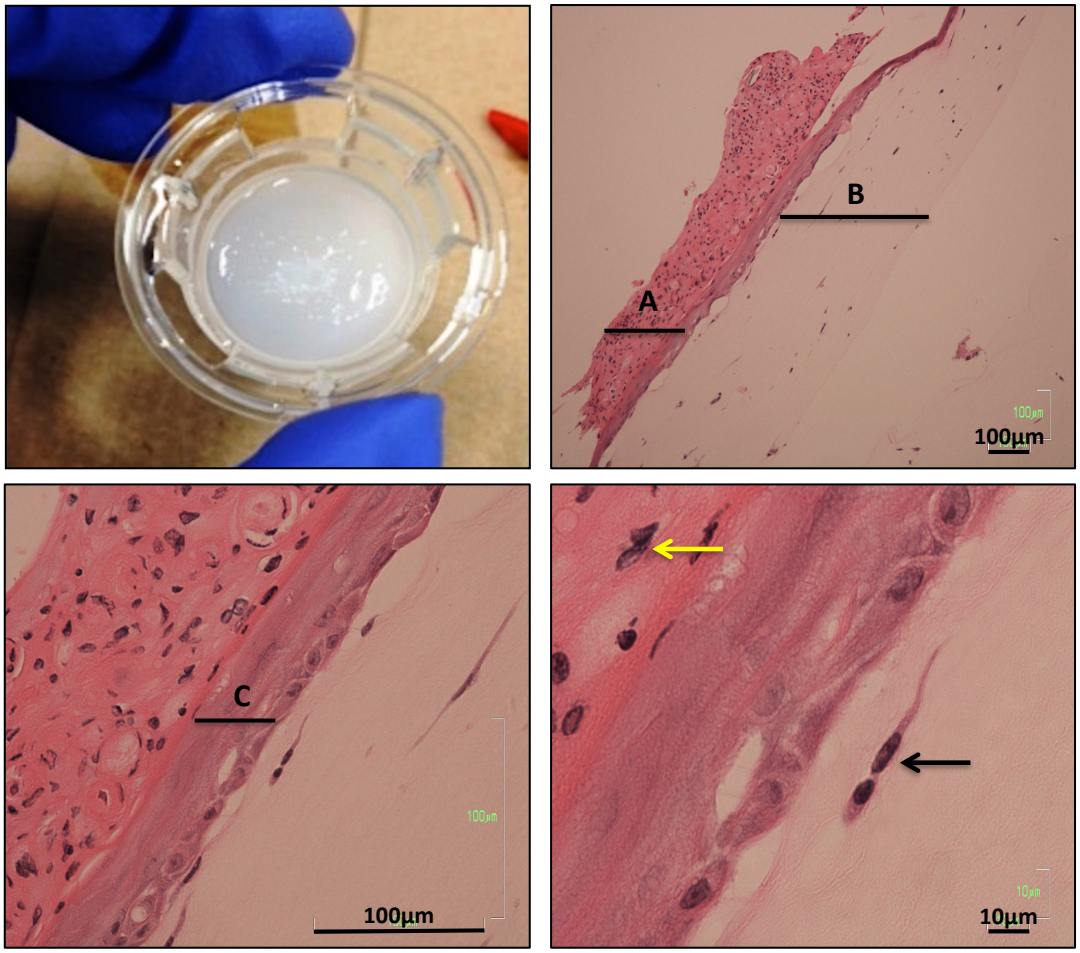

Figure 6.13 Images of a 3-dimensional human epidermal keratinocyte (HEKs) and human dermal fibroblast (HDFs) *in vitro* co-culture model.

The co-culture skin model was assessed histologically using the haematoxylin and eosin stain. The co-culture model contained an epidermal keratinocyte layer (**A**), fibroblast dermal layer (**B**) and basement membrane (**C**). A yellow arrow indicates the nuclei of the keratinocytes and fibroblasts are indicated using a black arrow. Images of the histology sections were taken at x10, x20 and x40 respectively.

6.4 Discussion

It was the aim of this chapter to assess the effect of several human chronic-wound-derived *S. aureus* and *P. aeruginosa* isolates, on fibroblast and keratinocyte scratch wound closure and cell viability. My study identified that *P. aeruginosa* BCM significantly affected HDF scratch closure and cell viability, whereas other treatment conditions such as *S. aureus* PCM and BCM and *P. aeruginosa* PCM had less of an effect.

Methicillin-resistant *S. aureus* (MRSA) PCM and BCM has been shown to cause a significant reduction in the migration of human dermal fibroblasts (Kirker et al. 2012). However *S. aureus*-conditioned medium had little effect on HDFs wound closure in this chapter. A possible explanation could be due to the fact that the *S. aureus* strains used in this chapter were methicillin-sensitive and therefore do not secrete the same soluble products that impede fibroblast migration. Indeed, differences between methicillin-sensitive *S. aureus* and MRSA, with regards to secreted virulence factors, has been reported (Schlievert et al. 2010). However a recent study by Souza and colleagues assessed the genes associated with virulence in methicillin-sensitive *S. aureus* and MRSA isolated from the surrounding environment and from equipment in a hospital, and found no significant differences in virulence factors, biofilm formation or cytokine production (Souza et al. 2014).

When repeating the scratch wound experiments using HEKs, it appeared that the HEKs were much more sensitive to bacterial-conditioned medium, to varying degrees, when compared to the HDFs. *In vitro* studies by Kirker and colleagues have previously demonstrated the detrimental properties of the soluble products of chronic wound-associated bacterial PCM and BCM on human epidermal keratinocytes. In the latter study, they found that the presence of PCM and BCM, from a single chronic wound-derived *S. aureus* isolate, significantly reduced normal human keratinocyte scratch closure and that *S. aureus* BCM significantly reduced keratinocyte viability and increased apoptosis when compared to *S. aureus* PCM (Kirker et al. 2009). Whilst this work emphasised the impact of biofilm-infected chronic wounds in terms of tissue destruction and reduced keratinocyte migration, it

was based upon the conditioned medium of a single wound-derived isolate. With this in mind, an interesting finding in the work presented in this thesis was the large variability in the effects of these clinical isolates on scratch closure. For instance, although the majority of *P. aeruginosa* BCM caused a significant reduction in wound closure in HDFs, BCM from a few isolates appeared to have little effect on cells in the scratch wound assay (data not shown).

It has been proposed that bacterial biofilms within a wound are the reason for increases in host proteases and therefore this proteolytic activity is indicative of a biofilm (Wolcott et al. 2008). The mechanism behind a biofilm-related increase in host proteases is currently unknown. In this chapter, it has been shown that *P. aeruginosa* BCM, but not PCM, elicits the release of a 56kDa metalloprotease from dermal fibroblasts. The soluble products of dermal fibroblasts treated with MRSA PCM and BCM was assessed for MMPs, growth factors and cytokines using ELISA and subsequently showed a significant increase in MMP-1 and MMP-3 secreted from MRSA PCM-treated fibroblasts, whereas MRSA BCM-treated fibroblasts appeared to suppress MMP-3 activity (Kirker et al. 2012). It was unclear as to whether this study explored other MMPs. Regarding the data in this chapter, an interesting observation was that of the 80kDa band detected in all controls and the *P. aeruginosa* PCM-treated supernatants, but not the *P. aeruginosa* BCM-treated supernatants. As outlined previously in this chapter, the soluble products of *P. aeruginosa* can effectively cleave other proteases (Twining et al. 1993). Therefore it is possible that the soluble products present in *P. aeruginosa* BCM, but not PCM, cleaved or potentially degraded this protease. I propose that the detection of the 56kDa protease in the *P. aeruginosa* BCM treatment group is a product of the HDFs in response to the bacterial-conditioned medium. However, an alternative explanation could be that soluble products released by the HDFs were able to cleave the bacterial protease to release an active 56kDa protease that could be successfully inhibited by EDTA. Indeed, the *P. aeruginosa*-derived bacterial protease was still observed in the EDTA-treated zymogram (see **Figure 6.4, B**), however the bands appeared slightly faint when compared to *P. aeruginosa* BCM alone, which may indicate potential

interactions between the bacterial protease and the soluble products of HDFs (see **Figure 6.4, A**).

An interesting finding in this chapter was the effect of the *P. aeruginosa*-derived partially purified proteases on HDFs and HEKs. This protease preparation using ammonium sulfate precipitation followed by dialysis, was referred to as 'partially purified' as there is more than one bacterial protease (62kDa and 52kDa). The use of SDS-PAGE has shown additional proteins in this preparation, however due to its high protease activity and the presence of protease bands in zymography (see **Chapter 5, Figure 5.8**), it was used to investigate its effects on wound closure. It was clear throughout the entirety of this project that the fibroblasts were more robust than the keratinocytes in culture, therefore it is not entirely surprising that the HEKs were more sensitive to the partially purified proteases than the HDFs. However considering this, I hypothesise that these proteases released by the biofilm within a chronic wound may reduce keratinocyte viability and prevent migration across the wound, but that these proteases do not effect dermal fibroblast migration. Rather, other soluble factors, possibly known *P. aeruginosa* virulence factors, may play more of a role in fibroblast migration and viability in biofilm-infected chronic wounds.

Whilst the use of monolayer scratch wounds can give a basic understanding of how a single cell type may regulate the secretion of MMPs in response to bacterial-conditioned medium, they simply do not mimic the complexity of human skin. It has been shown that the assessment of wound healing in 3-D fibroblast-keratinocyte skin equivalent models is comparable to that of wound healing in mice (Maione et al. 2014). It was therefore an important aim within this chapter to develop a 3-D human keratinocyte and fibroblast co-culture skin model that would serve as a more physiologically relevant model compared to that of single cell-type monolayers. The development of this model was based upon the model published previously, whereby fibroblasts were cultured in a 3-D fibrin gel matrix, followed by the culture of keratinocytes on the surface, to effectively reproduce the epidermal and dermal layer of the skin (Holland et al. 2008). Using this model, I had planned to test the effects of bacterial-conditioned medium, *P. aeruginosa*-derived partially purified proteases and low cell numbers of bacteria on unwounded and wounded skin, on the

expression of MMPs in the epidermal and dermal layers. A preliminary experiment developing this model resulted in a particularly acellular dermal layer. Therefore, further attempts were made to increase the seeding density of fibroblasts in the 3-D fibrin matrix, which resulted in contraction of the fibrin gel in culture (images not shown). However due to difficulties with keratinocyte cell culture on the fibrin gel, further attempts at the skin model were not progressed. Unfortunately it is unknown whether the encountered technical issues were due to the keratinocytes themselves or an incompatibility with the fibrin matrix. Although collagen matrices have always been the preferred scaffold of choice in these skin equivalent models, it has been known for some time that fibrin helps to promote keratinocyte migration and differentiation (Geer and Andreadis 2003). Furthermore Holland and colleagues successfully cultured this fibrin-containing skin equivalent for subsequent testing; however they used epidermal keratinocytes from neonatal foreskin rather than keratinocytes from adult epidermis, as seen in this chapter.

6.4.1 Limitations

It is important to note that the effects of bacterial-conditioned medium on cells may not reflect the potential effects of a biofilm incorporated into the scratch assay. This could be due to the fact that the PCM and BCM are produced in an isolated environment whereby factors affecting the bacterial growth include temperature, nutrient availability and oxygen. Such factors such as host cell-derived growth factors, cytokines, and proteases are not present in this artificial laboratory setting. Therefore it is logical to consider that these factors may have an impact on biofilm growth and maturity, and in particular, the release of soluble factors such as extracellular bacterial proteases and other virulence factors. It was indeed the intention of this particular study, to incorporate biofilms into the assay, however there was a lack of availability in 0.2µm cell culture inserts in the UK, and shipping from other countries from particular manufacturers was difficult and delayed. An alternative option to combat this was to grow the biofilm on the 0.2µm membrane filter before suspending it in a BD deep-well plate, whereby the cells were adhered

to the bottom of the well. However due to the nature of experiment set-up, involving the movement of the experiment to image cells and collect medium, there was a high rate of contamination of the culture medium, which caused cell death. It was also observed that the biofilm, once suspended on the surface of the medium in the BD deep-well plate, became unstable, with bacteria contaminating the culture medium in the well. Therefore it was decided to produce BCM without the presence of fibroblasts or keratinocytes in the well, with the intention to then treat cells separately.

6.4.2 Future Directions

Work performed in this chapter involved investigations into whether the expression of specific MMPs at a transcriptional level was altered after treatment with bacterial-conditioned medium. This initial work indicated that the expression of the MMPs' under investigation was not significantly altered. These results perhaps indicate that to focus solely on the expression of MMPs could be misleading, and that the use of techniques such as ELISA and substrate zymography, which focus on the detection of the secreted active protease, is more appropriate. Therefore, future work should investigate the levels of active host-derived MMPs in response to bacterial-conditioned medium in a more quantitative manner, possibly through the use of ELISA. Unfortunately in this study, the use of reverse zymography to identify tissue inhibitors of metalloproteases (TIMPs) was not possible due to technical difficulties and therefore future work would also aim to assess the presence of secreted TIMPs from both fibroblasts and keratinocytes in the scratch assay. It would also be more physiologically relevant to study bacterial-induced protease release from keratinocytes and fibroblasts taken from human chronic wounds (as done for the equine studies in Chapter 4 of this thesis).

An important observation in the results obtained from the work performed in this chapter is the variability between the clinical isolates and their effect on HDFs and HEKs. In particular it was noted that although the majority of bacterial-conditioned medium had detrimental effects on HEKs scratch wound closure and viability, not all

conditioned medium from these isolates had this effect. However, as the results are presented as a mean of the biological replicates, it is easy to overlook these anomalies. Questions do arise as to why the secreted products of these particular strains of bacteria do not have the same effect on cells as the majority of the isolates. Therefore it would be interesting to investigate the effect of factors such as the host wound environment in which the bacterial strain was isolated and the treatment strategies that were undertaken at the time of isolation, for example the use of antibiotics. It is possible that these factors may play a role in the virulence of these bacteria, which has a lasting effect that is continued through to laboratory culture.

6.4.3 Key Points

The major findings within this chapter include the significant reduction in scratch wound closure and cell viability of fibroblasts after treatment with the BCM of human chronic wound-derived *P. aeruginosa*. Furthermore, keratinocytes appeared much more sensitive to bacterial-conditioned medium, with varying effects on scratch closure and keratinocyte viability. Zymography revealed the release of a 56kDa metalloprotease from HDFs after *P. aeruginosa* BCM treatment, which was not detected in *P. aeruginosa BCM* or untreated fibroblast-conditioned medium alone. The expression of fibroblast MMP-1 and MMP-2 at a transcriptional level did not significantly alter when compared to the untreated control. However there was a significant decrease in the expression of collagen-I after treatment with *P. aeruginosa* BCM. The partially purified *P. aeruginosa*-derived proteases did not cause any significant reduction in fibroblast scratch closure, however 100µg of the partially purified protease caused a significant reduction in fibroblast viability at 48 hours. Interestingly, the *P. aeruginosa*-derived partially purified proteases had a significant effect on HEKs scratch closure and cellular viability, which would suggest that there is a detrimental effect of bacterial proteases on keratinocyte migration and cell viability in chronic wounds.

Chapter 7: Conclusions and Perspectives

7 Conclusions and Perspectives

The cellular and molecular mechanisms of physiological wound healing have been well researched, however there still remains a clear lack of understanding into the pathological mechanisms behind hard-to-heal chronic wounds. Theories exploring chronic wound pathology include the increased secretion of host-derived proteases and the presence of bacterial biofilms within the wound (Bjarnsholt et al. 2008, Wysocki et al. 1993). There are a plethora of advanced technology wound dressings and techniques such as debridement and antibiotic therapy that are used in the attempt to control wound exudate and microbial bioburden. Despite this, chronic wounds still remain an economic burden and health concern. Wound management appears to adopt a 'one-fits-all' approach, with no clear strategy employed by health organisations. One reason for this could be due to the lack of point-of-care tests available that may help decide the course of treatment for individual patients. In order to address this issue, research must be focussed on understanding the dynamics of the chronic wound. The research in this thesis focuses on understanding the potential role of bacterial biofilms and bacterial proteases in chronic wound pathology in horse and human.

Due to the clinically characteristic proteolytic environment of human chronic wounds and the presence of bacterial biofilms in both human and equine chronic wounds, I hypothesised that bacteria within these wounds contribute to an exaggerated proteolytic environment through the secretion of extracellular bacterial proteases and that both bacteria (in planktonic and biofilm form) and bacterial proteases may affect wound closure. Therefore the main objective of this thesis was to evaluate the proteolytic activity of bacteria isolated from equine and human chronic wounds in planktonic and biofilm form, and determine whether these bacteria reduced wound healing and affected fibroblast protease secretion *in vitro*.

The data presented in this thesis strongly supports the theory that bacteria, particularly *P. aeruginosa* in biofilm form, contribute to the protease-rich environment in human chronic wounds. Furthermore, it is likely that the presence of a bacterial biofilm contributes to the release of proteases from resident fibroblasts in

human chronic wounds (see **Figure 7.1**). The reader is directed to **Table 7.1** for an overview of all bacterial- and fibroblast-derived proteases identified in this thesis.

The exaggerated protease-rich environment subsequently leads to the excessive degradation of extracellular matrix (ECM) components and potentially provides additional nutrients for a developing biofilm. Indeed, a proteomic analysis of catheter-associated biofilm has shown the release of *P. aeruginosa*-derived proteases, which are not only thought to play a role as virulence factors in the evasion of host immune responses but also play a role in nutrient acquisition through amino acid metabolism (Lassek et al. 2015). Interesting findings relating to this concept include the significant difference in protease activity between human-derived *P. aeruginosa* in planktonic and biofilm form (see **Figure 7.2**) and the presence of a secreted protease that could only be detected in biofilm cultures. Few studies have focussed on biofilm-specific secreted proteases. According to a study by Whiteley and colleagues, there are a small but significant percentage of differentially expressed genes in planktonic and biofilm *P. aeruginosa* (Whiteley et al. 2001). A laboratory strain of *P. aeruginosa* has been shown to secrete a 25kDa metalloprotease named Mep72, which is only secreted from the biofilm form and not planktonic cells (Passmore et al. 2015). Future work would aim to investigate whether the secretion of these proteases are linked to the development of *P. aeruginosa* biofilms in chronic wounds. If indeed they are, it may be interesting to consider this mechanism as a therapeutic target, possibly for the development of a diagnostic point-of-care test that indicates the presence of a biofilm within a wound. Alternatively, this protease could be pharmacologically targeted to disrupt biofilm formation in chronic wounds.

The bacterial-derived extracellular proteases detected in human-derived *P. aeruginosa* BCM were partially purified, however the results in Chapter 6 of this thesis show that these proteases did not significantly affect *in-vitro* human dermal fibroblast (HDF) wound closure. Although the bacterial-derived proteases did significantly reduce human epidermal keratinocyte (HEK) wound closure, which may suggest a potential role for bacterial proteases in the prevention of re-epithelialisation, these results should be approached with caution. *In vitro* culture of the HEKs was problematic throughout this study due to cell death and a lack of

cellular proliferation, despite obtaining several different batches of these cells from the company from which they were purchased. Thus, more research is needed to elucidate the effects of bacterial proteases on keratinocyte wound closure by assessing other available keratinocytes such as that of a neonatal origin, which are considered more robust in culture. Investigating the role of this bacterial protease, for example, as a virulence factor in evading host immune responses, would be an essential focus of future work.

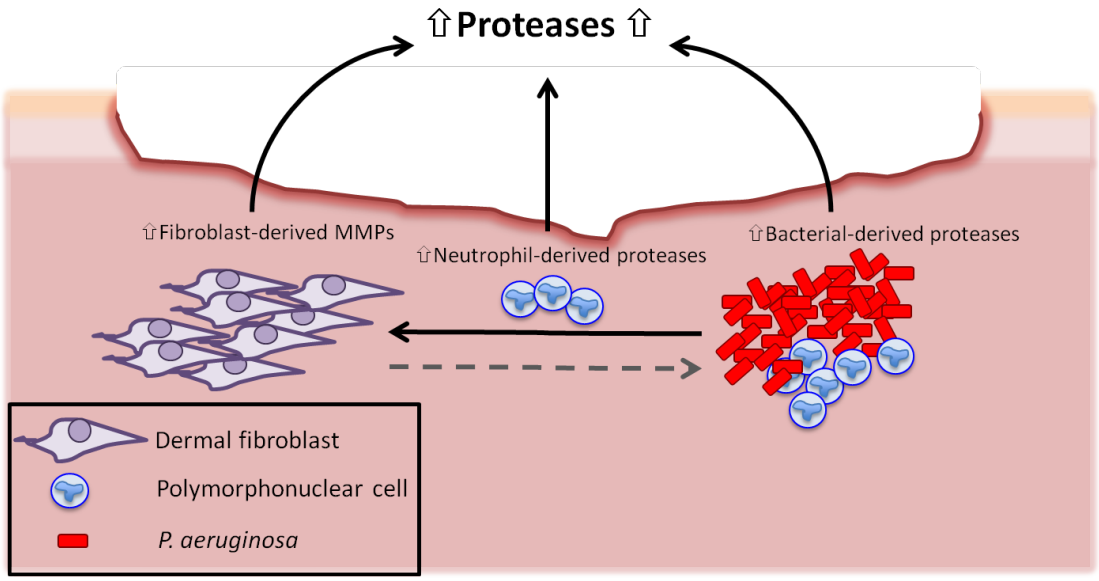

Figure 7.1 Representation of the potential sources of protease release, which may contribute to the exaggerated proteolytic environment of human chronic wounds.

It is hypothesised that bacterial biofilms, particularly *P. aeruginosa*, contribute to the excessive production of proteases in chronic wound pathology through the release of extracellular bacterial proteases. Furthermore the presence of a *P. aeruginosa* biofilm within the wound may induce the release of matrix metalloproteases (MMPs) from resident dermal fibroblasts. It is unknown whether fibroblast-derived MMPs effect the production of extracellular bacterial-derived proteases (indicated by dashed grey arrow).

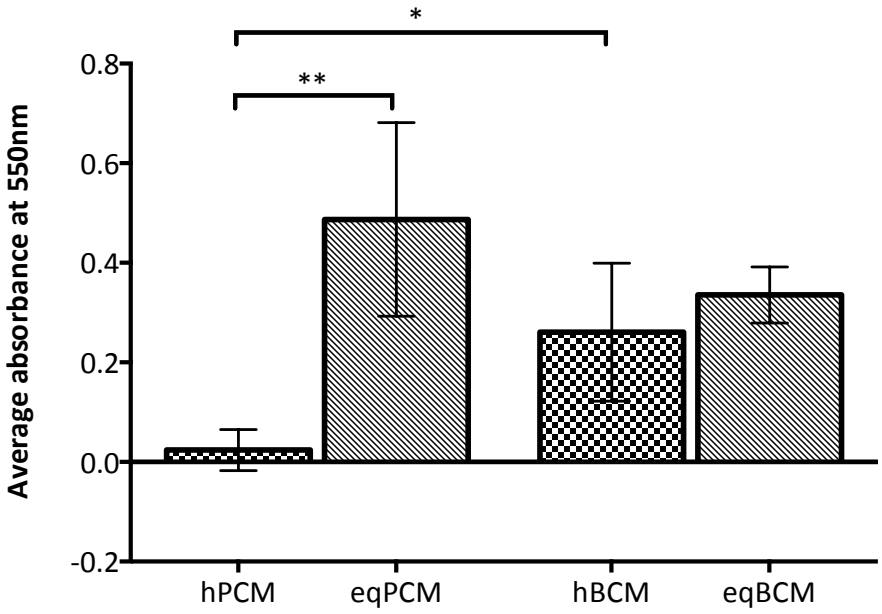

Figure 7.2 A comparison between the proteolytic activity of human and equine *P. aeruginosa* clinical isolates in the Azocoll assay.

Abbreviations include planktonic-conditioned medium (PCM), biofilm-conditioned medium (BCM), human (h) and equine (eq). Error bars represent standard deviation. Statistical analysis was performed using the Kruskal-Wallis non-parametric ANOVA with a Dunn's multiple comparison test. Statistical significance is represented as *P <0.01; **P <0.0001.

	Detected proteases	
Source of bacteria	*P. aeruginosa*-derived	Fibroblast (host)-derived
Equine chronic wounds	62kDa, 52kDa, 42kDa	57kDa, 38kDa
Human chronic wounds	62kDa, 52kDa	56kDa

Table 7.1 An overview of the bacterial- and fibroblast-derived proteases detected within the study.

Dulbecco's modified Eagle's medium (DMEM) was used to obtain bacterial- and fibroblast-derived conditioned medium. Proteases were either detected using gelatin or collagen zymography. Molecular weights were estimated using a molecular weight marker.

As mentioned previously within this thesis, the study of equine chronic wounds is gaining precedent due to the parallels that can be drawn to human skin disorders, particularly fibroproliferative disorders. In the context of equine chronic wounds, whereby exuberant granulation tissue is present, it is unclear whether there is a protease-rich environment similar to that diagnosed in human chronic wounds. The available literature merely draws assumptions based on the clinical findings in human chronic wounds. Therefore it must be acknowledged that the clinical characteristics of equine and human wounds do differ, with equine wounds often presenting with exuberant granulation tissue unlike human chronic wounds.

Whilst the findings in this thesis show that equine-derived *P. aeruginosa* secrete high levels of extracellular proteases in planktonic and biofilm form, these proteases may not effect the production of granulation tissue. Granulation tissue fibroblasts (GTFs) showed faster wound closure and appeared to be more resilient towards the effects of bacterial-conditioned medium compared to the normal fibroblasts (NFs). Although the presence of *P. aeruginosa* BCM still caused a significant reduction in GTF wound closure, these observations may indicate that GTFs within equine chronic wounds are

better at withstanding the soluble products released by the biofilm. Further studies into the effects of biofilms on the proliferative capacity and viability may help to clarify this. The presence of *P. aeruginosa* in biofilm form also elicited the secretion of proteases from NFs but not from GTFs. In light of this, future work would involve the investigation of tissue inhibitors of metalloproteases (TIMPs) released from NFs and GTFs and other detectable bacterial-derived inhibitors may help gain insight into overall proteases imbalances in equine chronic wounds.

When reflecting upon the equine and human data as a whole, the discovery that human-derived *P. aeruginosa* in planktonic form produce low levels of protease activity was indeed a surprise, considering the high levels secreted by planktonic equine-derived *P. aeruginosa* (see **Figure 7.2**). Whilst variations in proteolytic activity between varying strains is to be expected, it still unearths the question of whether there is a relationship between the environment from which the isolate originates and its protease activity. Horses are exposed to a plethora of environmental microbes, which may have an effect on the protease profile of these individual isolates. Very few studies have addressed this issue of mixed species biofilms and how the bacterial secretome differs within this environment. One study by Purschke and colleagues identified a uniquely expressed collection of proteins specific to *P. aeruginosa* and *Candida albicans* in a mixed species biofilm (Purschke et al. 2012). More specifically, they determined that the bacterial secretome was less diverse in a mixed-species biofilm, however *P. aeruginosa*-derived proteins relating to iron-acquisition (a process that is thought to be necessary for biofilm formation), such as pyoverdine, was significantly increased in mixed-species biofilms. Interestingly, mass spectrometry results also showed a decrease in detected *P. aeruginosa* AprA, LasA and LasB proteases in mixed-species biofilms (Purschke et al. 2012). Indeed, one of the limitations of the research within this thesis is the determination of the proteolytic activity of the bacterial isolates in an artificial environment, whereby individual species lack interactions with other microorganisms that may have been present within the wound. It would be interesting to investigate further with mixed species biofilms, *P. aeruginosa* and *S. aureus*, to determine how protease secretion is affected, providing more clinically relevant data.

Similarities in the proteases detected in equine- and human-derived *P. aeruginosa*-conditioned medium and the medium from *P. aeruginosa*-treated fibroblasts can be seen (see **Table 7.1**). Both equine- and human-derived *P. aeruginosa* secreted a 62kDa and 52kDa proteases (respectively with variation between biological replicates). Furthermore it can be argued that equine and human fibroblasts secrete the same protease in response to the *P. aeruginosa* biofilm, with proteases of 57kDa and 56kDa detected, respectively. A notable observation in the detection of both equine- and human-derived bacterial proteases was the resistance of these proteases to broad-spectrum inhibitors. Unfortunately due to this and a lack of conclusive mass spectrometry data (Chapter 3), it was difficult to determine the identification of these proteases. Nevertheless, if the inhibition of extracellular bacterial proteases were to be considered as a target to discourage biofilm development, the potential resistance of these proteases to protease inhibitors may prove problematic.

To conclude, the role of *P. aeruginosa* biofilms and their extracellular proteases in equine chronic wound pathology remains undetermined. Whilst this is the first study to demonstrate high levels of extracellular proteases in the planktonic and biofilm forms of equine-derived *P. aeruginosa*, it is difficult to relate the clinical characteristics of equine wounds with this bacterial physiology. Understanding the role of protease inhibitors such as TIMPs in equine chronic wounds may be of great clinical significance. Nevertheless, the results within this thesis do help to elucidate the detrimental impact of *P. aeruginosa* biofilms in human chronic wound pathology, with high levels of secreted proteases from these biofilms and the presence of these biofilms relating to the secretion of host-derived proteases, thus contributing to an exaggerated proteolytic environment. For the first time, this research has shown the presence of a 62kDa and 52kDa *P. aeruginosa* protease that appears to be specific to biofilm forms and not planktonic forms. However further research needs to be done in order to understand the role of this specific *P. aeruginosa* protease in the development of the biofilm and the pathogenesis of human chronic wounds.

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Published Abstracts

2014:

Biofilms And Bacterial Proteases: The Effect On Wound Closure And Host Protease Production. European Tissue Repair Society (ETRS) Edinburgh, UK; September 2014. Oral presentation.

The Effect Of Chronic Wound-Derived Bacteria On Wound Closure And Host Protease Production *In Vitro*. European Wound Management Association (EWMA), Madrid, Spain; May 2014. Poster Presentation.

Chronic Wound-Derived Bacteria In Biofilm Form Shows High Levels Of Proteolytic Activity. EWMA, Madrid, Spain; May 2014. Poster Presentation.

Proteolytic Activity of Chronic Wound-derived Bacteria: Planktonic vs Biofilm. Symposium of Advanced Wound Care/ Wound Healing Society (SAWC/WHS), Orlando, Florida, USA; April 2014. Oral Presentation.

Chronic Wound Bacterial Isolates, Their Effect on Wound Closure and Host Protease Production *In Vitro*. SAWC/WHS, Orlando, Florida, USA; April 2014. Poster Presentation.

2013:

P. aeruginosa Isolated from Chronic Wounds Secrete Proteases That Impair Wound Healing – An In Vitro Study. EWMA, Copenhagen, Denmark; May 2013. Poster Presentation.

The Effect of Novel Biofilm Technologies on Planktonic and Biofilm Forming Microorganisms – *In Vitro*. EWMA, Copenhagen, Denmark; May 2013. Poster Presentation.

The Assessment of Potential Biofilm-Disrupting Technologies *In Vitro*. EuroBiofilms, Ghent, Belgium; September 2013. Poster Presentation.

2012:

Modulating Effect of a Novel Natural Fibre Dressing on Protease Activity in Chronic Wounds. EWMA, Vienna, Austria; May 2012. Poster presentation.

Bacteria Isolated From Chronic Equine Wounds Produce High Levels of Protease Activity. EWMA, Vienna, Austria; May 2012. Poster Presentation.

Appendix I: Protease activity in equine *P. aeruginosa* planktonic-conditioned medium (PCM) using milk-casein agar

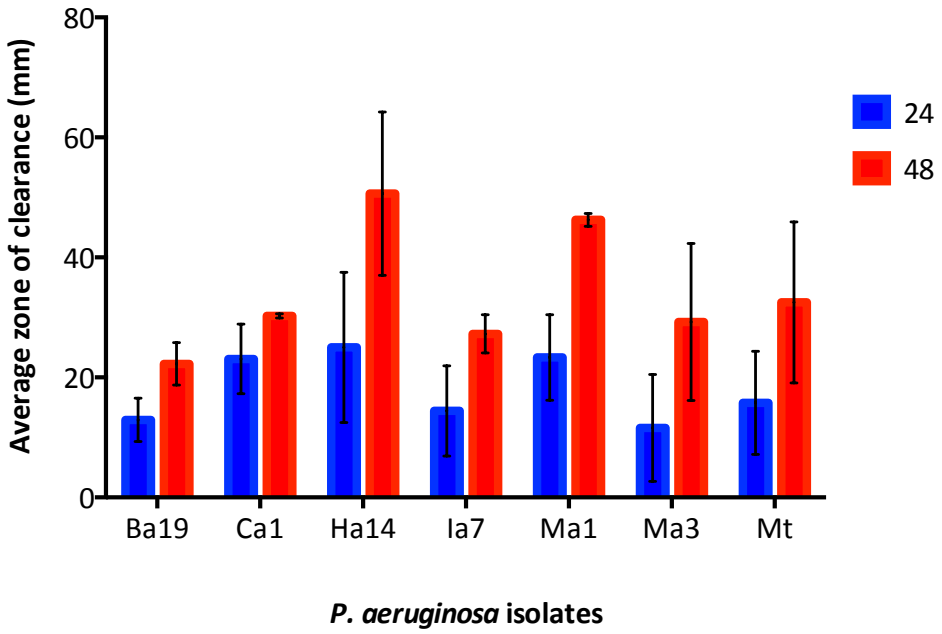


Figure I A Protease activity of equine chronic wound-derived *P. aeruginosa* using milk-casein agar. *P. aeruginosa* planktonic-conditioned medium (PCM) was added to milk-casein agar and incubated at 37°C. Zones of clearance in milk-casein agar was measured at 24- and 48-hours. Error bars represent standard deviation. The experiment was repeated three times (n=3).

Appendix II: The Sequestration of MMP-2 using a Novel Natural Fibre Dressing and the Assessment of Potential Biofilm-Disrupting Technologies *In Vitro* - Work proposed by Advanced Medical Solutions Plc

Introduction

Since the identification of elevated proteases in chronic wounds (Wysocki et al. 1993), wound care has been directed toward the management of excessive chronic wound fluid in order to control the destructive action of potential tissue-degrading MMPs. For instance, diagnostic tools such as WOUNDCHEK™ Protease status (WOUNDCHEK™ Laboratories, UK), a novel point of care test to detect elevated protease activity in hard-to-heal chronic wounds, has been developed to create a more targeted approach to wound management. Furthermore, a range of wound dressings with high-absorption qualities are used to control MMP-rich wound exudate. As mentioned in Chapter 1 of this thesis, materials such as alginates, hydrofiber, foam, hydrogel and hydrocolloid, all possess absorptive properties and are commonly used materials for wound dressings. Dressings incorporated with a substrate such as collagen, have also been designed to provide an additional substrate for excess MMP's, as well as for bacterial collagenases that have also been detected within wounds (Metzmacher et al. 2007). In addition there has been much attention surrounding biofilms and chronic wound pathology and subsequently, wound care companies have developed wound care products with not only high absorptive properties, but also antimicrobial technology. A good example of this is wound dressings incorporated with silver, which have been shown to reduce bacterial numbers in chronic wound-associated bacteria, with a low risk of microbial silver resistance (Percival et al. 2007, Percival et al. 2008). The use of silver nanoparticle technology is also being investigated and has been shown to reduce bacterial cell numbers in an *in vitro Pseudomonas aeruginosa* biofilm model, and, with human fibroblast biocompatibility, is thought to be able to reduce microbial load and improve wound closure in chronic wounds (Velázquez-Velázquez et al. 2014). Manuka honey in wound management has also shown promising results, with

significant evidence of wound healing in methicillin-resistant *Staphylococcus aureus* (MRSA) infected wounds that have been managed with Manuka honey dressings (Visavadia et al. 2008). Some research groups have focussed on developing wound dressings that can control both elevated MMPs and control wound bioburden. Adhirajan and colleagues developed a novel biomaterial consisting of an MMP inhibitor, 2,3-dihydroxybenzoic acid, and gelatin microsphere conjugate, loaded with the antmicrobial doxycycline and incorporated into a collagen scaffold (Adhirajan et al. 2009). This novel biomaterial effectively reduced MMP-2 and MMP-9 released from diabetic wound tissues, displayed biocompatibility with human dermal fibroblasts *in vitro* and demonstrated biocidal activity against *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *P. aeruginosa* laboratory strains.

This chapter aims to assess the MMP sequestration of a novel natural fibre dressing produced by Advanced Medical Solutions Plc (project funder) in comparison with commercially available wound dressings, including a collagen-substrate dressing. These dressings will be tested for their efficacy in the absorption of MMP-2, shown to be upregulated in human chronic wounds, *in vitro*, which will be assessed using gelatin zymography. This chapter also aims to assess potential biofilm disrupting technologies against *P. aeruginosa*, *S. aureus* and *Candida albicans* biofilms *in vitro*, in order to determine their antimicrobial efficacy for incorporation into wound dressings. More specifically, the technologies to be tested include a chelating agent, a commercial used polymer antimicrobial, non-ionic surfactants and a commercially available pre-blended antimicrobial.

Materials and Methods

For a detailed description of the materials and methods used in this chapter, please refer to Chapter 2 of this thesis (2.4 Biofilm-disrupting technologies study, 2.11 MMP-2 isolation, 2.12 Wound dressing studies: Sequestration of MMP-2, 2.18 Statistical analysis).

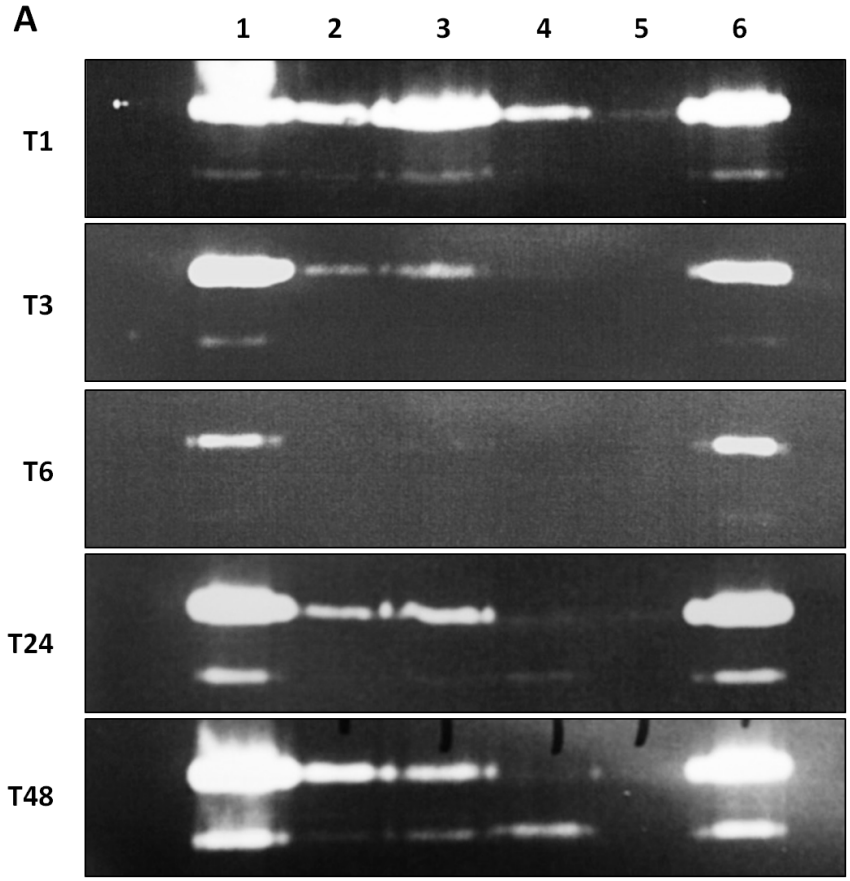
Results

Sequestration of pro-MMP-2 and active MMP-2 by wound dressings

The ability of wound dressings to sequester secreted host-derived proteases that are present in the wound environment could prove advantageous in wound management by reducing protease-related local tissue degradation. Therefore, the ability of a dressing to sequester MMP-2 was tested in several commercially available wound dressings, including a hydrofibre dressing, an alginate dressing, collagen substrate dressing and hydrocolloid dressing. In addition, a novel, natural fibre prototype wound dressing, manufactured by Advanced Medical Solutions Plc was also tested.

After 1 hour, the collagen-substrate dressing caused a significant reduction in detectable pro-MMP-2 and active MMP-2 ($P < 0.001$ and $P < 0.05$) respectively, with values of 3.4% ± 2.3% (standard deviation) and 32.1% ± 6.2% when compared with the MMP-2 control (100%) (see **Figure II-A**). Furthermore, the novel natural fibre dressing caused a reduction in detectable pro-MMP-2 to 17.8% ± 9.1% and active MMP-2 to 32.7% ± 27.9% ($P < 0.01$ and $P < 0.05$) and hydrofibre dressing caused a significant reduction in pro-MMP-2 to 27.2% ± 10.6% ($P < 0.01$) but not active MMP-2. The alginate and hydrocolloid dressing did not significantly reduce detectable MMP- 2 in pro-enzyme and active forms after 1-hour incubation. After 3 hours incubation, pro-MMP-2 and active MMP-2 bands were undetectable after incubation with the collagen-substrate dressing with values of 9.5% ± 9.7% and 9.1% ± 6.7% ($P < 0.001$) respectively. The novel natural fibre dressing also caused a reduction in pro-MMP-2 and active MMP-2, with values of 12.2% ± 10.1% and 17.3% ± 13.5% ($P < 0.001$ and $P < 0.01$) respectively. The hydrofibre and alginate dressings caused a significant reduction in pro-MMP-2 to 8.7% ± 7.6% and 26.0% ± 16.0% ($P < 0.001$ and $P < 0.01$), with hydrofibre reducing active MMP-2 to 5.2% ± 1.8% ($P < 0.001$) but the alginate dressing having no significant effect on detectable active MMP-2. After 6 hours, hydrofibre, the novel natural fibre dressing, the collagen-substrate dressing and the alginate dressing significantly reduced detectable pro-MMP-2 to 6.3% ± 2.4%, 10.9% ± 0.6%, 6.4% ± 2.9% ($P < 0.001$) and 25.5% ± 8.0% ($P < 0.01$) respectively.

Similarly, the hydrofibre, the novel natural fibre dressing, the collagen-substrate dressing and the alginate dressing significantly reduced active MMP-2 to 4.4% ± 2.6%, 2.2% ± 1.3%, 4.8% ± 4.1% ($P < 0.001$) and 14.2% ± 10.7% ($P < 0.01$). After 24 hours, the efficacy of the hydrofibre and alginate dressings to reduce detectable MMP-2, appeared to diminish, however percentage values still showed a significant reduction in pro-MMP-2 to 10.4% ± 7.8% and 10.7% ± 3.6% ($P < 0.001$) and a significant reduction in active MMP-2 to 2.6% ± 0.9% and 9.8% ± 6.1% ($P < 0.001$). The collagen-substrate dressing continued to maintain low levels of pro-MMP and active MMP-2, with values of 2.5% ± 2.7% and 1.9% ± 2.0% ($P < 0.001$). The novel natural fibre dressing also maintain reduced detectable pro-MMP-2 levels of 2.8% ± 2.0% ($P < 0.001$) and active MMP-2 of 14.3% ± 4.1% ($P < 0.01$). After 48 hours, sustained reductions, to varying degrees, in pro-MMP-2 and active MMP-2 was detected in the hydrofibre dressing [25.3% ± 11.7% ($P < 0.05$) and 4.6% ± 2.3% ($P < 0.01$)], the alginate dressing [7.0% ± 4.7% and 8.3% ± 8.7% ($P < 0.01$)], the novel natural fibre dressing [2.2% ± 0.1% ($P < 0.01$) and 22.8% ± 8.6% ($P < 0.05$)] and the collagen-substrate dressing [4.8% ± 1.5% and 0.6% ± 0.1% ($P < 0.01$)]. The hydrocolloid dressing was ineffective in the sequestration of MMP-2 and showed no significant reduction in pro-MMP-2 and active MMP-2 at all time points of the experiment. There were no significant differences between the collagen-substrate dressing and the novel natural fibre dressing.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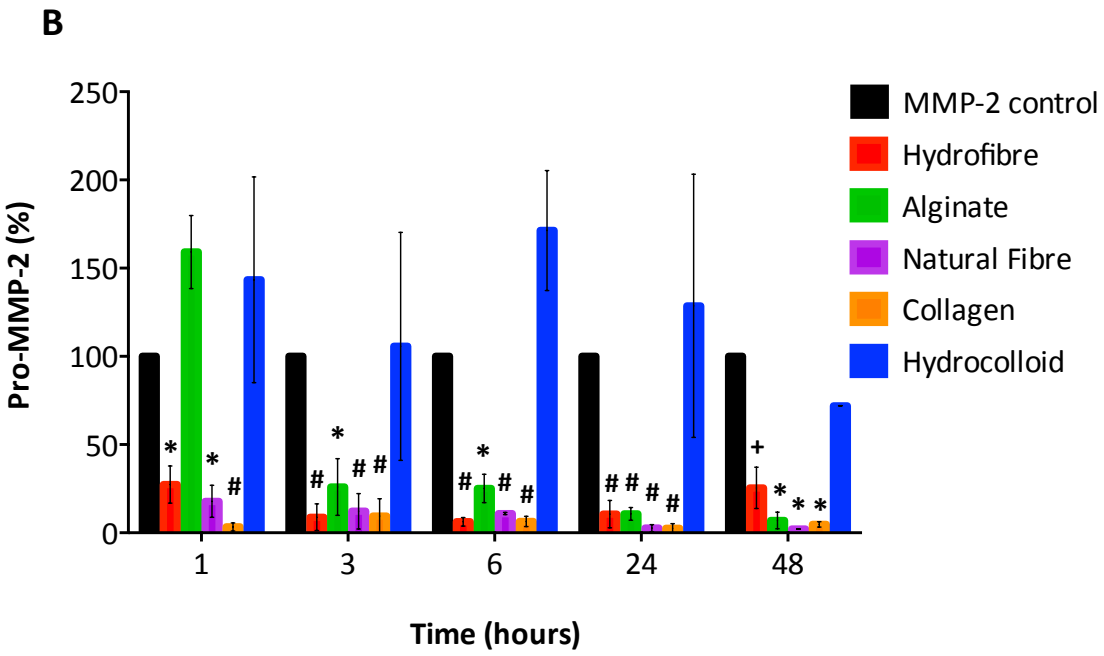

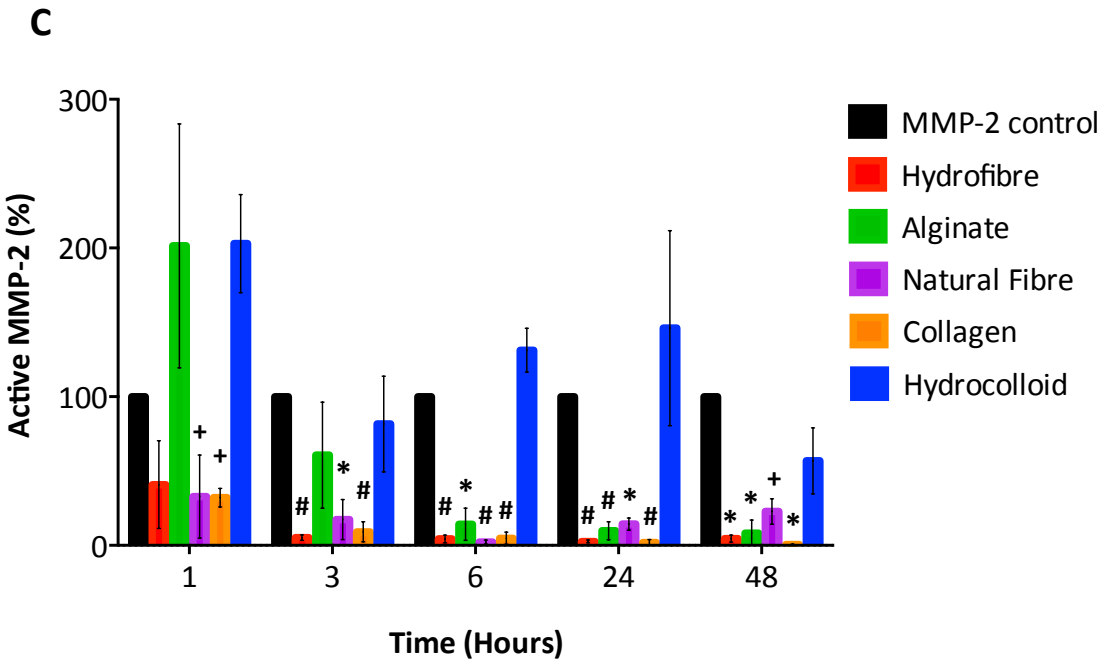

Figure II-A Sequestration of pro-MMP-2 and active MMP-2.

Purified equine fibroblast-derived MMP-2 was incubated with 0.1g of wound dressing for 1, 3, 6, 24 or 48 hours at 37°C, before washing the dressings in PBS and testing the supernatant for MMP-2 activity using gelatin zymography. Images of

gelatin zymograms showing MMP-2 activity following incubation with wound dressing was taken using the Syngene gel doc (**A**) and pro-MMP-2 (**B**) and active MMP-2 band intensity (**C**) was measured using GeneTools image analysis software. Lane 1 represents MMP-2 alone. Lanes 2-6 represent the hydrofibre, alginate, novel natural fibre dressing, the collagen-substrate and hydrocolloid dressings respectively. Values are presented as percentage (%) whereby MMP-2 alone is 100%. Error bars represent standard deviation. Statistical analysis was performed using a two-way analysis of variants (ANOVA) with a Tukey's multiple comparisons test. Statistical significance is represented as **+**P <0.05, *****P <0.01 and **#**P <0.001 when compared with the MMP-2 control. The experiment was repeated three times (n=3).

Biofilm forming potential of Staphylococcus aureus ATCC 9538, Pseudomonas aeruginosa ATCC 9027 and Candida albicans ATCC 10231

The *in vitro* crystal violet assay was used to test the biofilm forming potential of *Staphylococcus aureus* ATCC 9538, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231 at 24, 48 and 72 hours. Following crystal violet staining, it was clear that both *P. aeruginosa* and *S. aureus* formed dense biofilms *in vitro* when compared with *C. albicans* whereby crystal violet staining appeared very faint (see **Figure II-B**). Upon solubilisation of crystal violet-stained biofilms the absorbance was measured and it was determined that high absorbance values were represented high biofilm forming potential, due to the greater amount of solubilised crystal violet. Equally, low absorbance values represent low biofilm forming potential. Results showed significantly higher biofilm forming potential for *S. aureus* and *P. aeruginosa* when compared with *C. albicans* at 24, 48 and 72 hours (P <0.0001). No significant difference in biofilm forming potential between *S. aureus* and *P. aeruginosa* were detected at 24 and 72 hours. However, *S. aureus* showed significantly higher biofilm forming potential than *P. aeruginosa* at 48 hours (P <0.0001) (see **Figure II-C**).

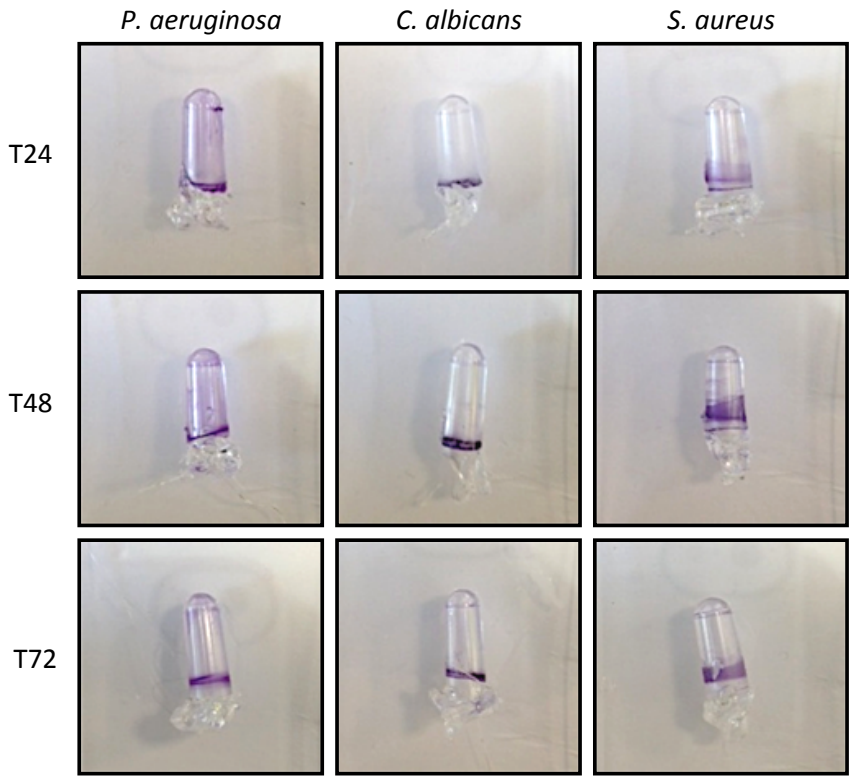

Figure II-B Representative images of biofilms stained with crystal violet.

P. aeruginosa, *C. albicans* and *S. aureus* were grown in a 96-well plate with peg-lid for 24-, 48- and 72-hours. After incubation, biofilm growth on the pegs was assessed using 0.5% crystal violet stain. Pegs were removed from the lid for the purpose of obtaining images only.

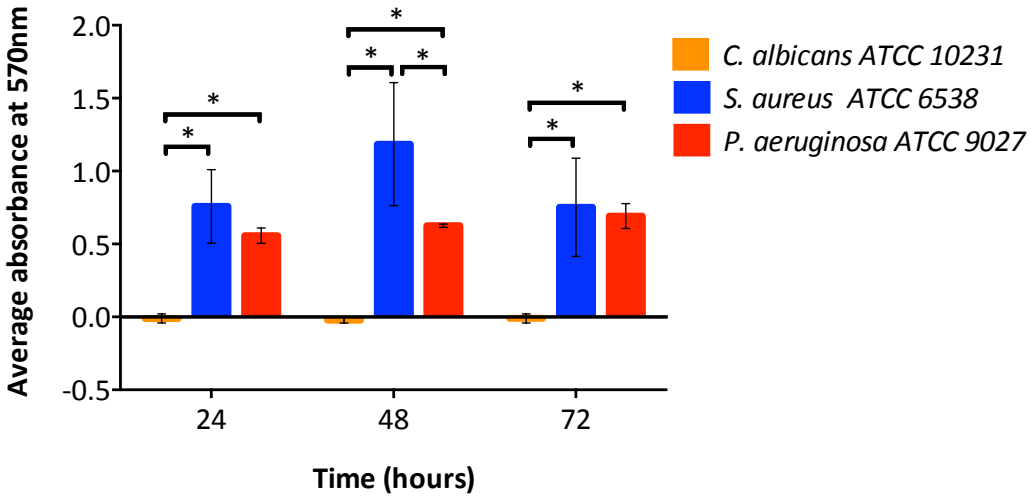

Figure III-C Biofilm forming potential of *S. aureus*, *P. aeruginosa* and *C. albicans* biofilms using crystal violet.

Untreated 24-, 48- and 72-hour biofilms were stained with 0.5% crystal violet for 15 minutes before biofilms were washed in tap water. Biofilms were air-dried and solubilised in 30% acetic acid. The absorbance of each sample was read at 570nm. Statistical analysis was performed using two-way analysis of variants (ANOVA) with a Tukey's multiple comparisons test. Statistical significance is represented as *P <0.0001. Error bars represent standard deviation. The experiment was repeated six times (n=6).

MIC and MBEC values of a chelating agent, surfactant A, surfactant B, polymer antimicrobial and commercial pre-blended antimicrobial on 24 hour biofilms

In this section of the chapter, potential biofilm-disrupting technologies including a chelating agent, surfactants A and B, a polymer antimicrobial and a commercially-available pre-blended antimicrobial, which is thought to contain a polymer antimicrobial, Benzalkonium chloride, surfactants and silicone siloxanes, was tested on *S. aureus* ATCC 9538, *P. aeruginosa* ATCC 9027 *and C. albicans* ATCC 10231. More specifically, the minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) was determined. MBC was defined as the

minimum concentration of antimicrobial that eradicates the growth of the dispersed, planktonic cells from the biofilm. MBEC was defined as the minimum concentration of antimicrobial that eradicates the biofilm.

In this study, the pre-blended antimicrobial effectively inhibited the growth of *P. aeruginosa*, *S. aureus* and *C. albicans* at low concentrations, with MBC values of 0.031%, 0.0075% and 0.0075% respectively (see **Table II-A**). MBEC results showed that higher a concentration of the pre-blended antimicrobial was needed to eradicate biofilm growth, with values of 0.063%, 0.015% and 0.015% for *P. aeruginosa*, *S. aureus* and *C. albicans*. In addition to this, the polymer antimicrobial successfully eradicated planktonic cells and biofilm *P. aeruginosa* at the same concentration as the pre-blended antimicrobial. However, higher concentrations of polymer antimicrobial were needed to eradicated *S. aureus* and *C. albicans* when compared with the pre-blended antimicrobial, with MBC/MBEC values of 0.031/0.031% and 0.015/0.015%. Surfactants A and B both failed to eradicate planktonic and biofilm growth in all microorganisms at the highest concentration tested in the assay (2%-2.5% w/v). The chelating agent also failed to inhibit *P. aeruginosa* and *S. aureus* growth at the highest concentration tested in the assay (2% w/v), however eradication of planktonic *C. albicans* was recorded, with a MBC value of 0.5%, however the highest concentration of the chelating agent failed to inhibit the *C. albicans* biofilm (see **Table II-A**).

Checkerboard analysis was used in order to test whether polymer antimicrobial in combination with either the chelating agent, surfactant A or surfactant B, reduced MBC and MBEC values, potentially indicating synergy. Combinations the potential biofilm disruptor with polymer antimicrobial lead to the eradication of both planktonic cells and biofilms in all microorganisms (see **Table II-B**), however this appeared to be due to polymer antimicrobial and not the addition of potential biofilm-disruptor, given that MBC and MBEC values for polymer antimicrobial alone and polymer antimicrobial in combination with a biofilm disruptor were the same. Nevertheless, the synergy of each combination, which can be determined by calculating the fractional biocidal concentration (FBC) and fractional biofilm eradication concentration (FBEC) (see Chapter 2, Materials and Methods), could not

be determined. This was due to MBC and MBEC values of the chelating agent, surfactant A and surfactant B alone being greater than the concentration tested. Exceptions were found with the combination of chelating agent/ polymer antimicrobial and surfactant A/ polymer antimicrobial, which revealed 'indifference' in the synergy of these substances for *C. albicans* (see **Table II-C**).

	Test substance (% w/v)									
	Pre-blended antimicrobial		**Polymer antimicrobial**		**SB**		**CA**		**SA**	
Microorganism	MBC	MBEC	MBC	MBEC	MBC	MBEC	MBC	MBEC	MBC	MBEC
P. aeruginosa ATCC 9027	0.031	0.063	0.031	0.063	>2	>2	>2	>2	>2.5	>2.5
S. aureus ATCC 6538	0.0075	0.015	0.031	0.031	>2	>2	>2	>2	>2.5	>2.5
C. albicans ATCC 10231	0.0075	0.015	0.015	0.015	>2	>2	0.5	>2	>2.5	>2.5

Table II-A Minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) for potential biofilm-disrupting substances on 24-hour biofilms.

Abbreviations include, SB = surfactant B; CA = chelating agent, SA = surfactant A. The experiment was repeated three times (n=3).

	Combinations of substances (% w/v)					
	SB/ polymer antimicrobial		CA/ polymer antimicrobial		SA/ polymer antimicrobial	
Microorganism	MBC	MBEC	MBC	MBEC	MBC	MBEC
P. aeruginosa ATCC 9027	0.031/ 0.031	0.063/ 0.063	0.125/ 0.031	2/ 0.063	0/ 0.015	0/ 0.063
S. aureus ATCC 6538	0.015/ 0.031	0.063/ 0.063	0.031/ 0.031	0.031/ 0.031	0/ 0.015	2.5/ 0.031
C. albicans ATCC 10231	0/ 0.015	0/ 0.015	0.5/ 0.015	0/ 0.015	0/ 0.015	0/ 0.031

Table II-B Minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) of the polymer antimicrobial combination-treated 24-hour biofilms: Results from checkboard analysis.

Abbreviations include, SB = surfactant B; CA = chelating agent, SA = surfactant A. The experiment was repeated three times (n=3).

	Substance combinations					
	SB and polymer antimicrobial		CA and polymer antimicrobial		SA and polymer antimicrobial	
Microorganism	FIC	FBEC	FIC	FBEC	FIC	FBEC
P. aeruginosa ATCC 9027	ND	ND	ND	ND	ND	ND
S. aureus ATCC 6538	ND	ND	ND	ND	ND	ND
C. albicans ATCC 10231	ND	ND	2.0 (Indifferent)	ND	2.0 (Indifferent)	ND

Table II-C Determination of synergy between the polymer antimicrobial and other potential biofilm disruptors.

Abbreviations include, FIC = Fractional inhibitory concentration; FBEC = Fractional biofilm eradication concentration; ND = Not Determined; SB = surfactant B; CA = chelating agent, SA = surfactant A. The experiment was repeated three times (n=3).

The effect of the polymer antimicrobial and the pre-blended antimicrobial on 48- and 72-hour biofilms

It is thought that biofilms within a chronic wound are established structures and therefore, the efficacy of the pre-blended antimicrobial and polymer antimicrobial to eradicate biofilms grown for 48 and 72 hours was tested. In this portion of the study, much lower concentrations of polymer antimicrobial and the pre-blended antimicrobial were tested. Subsequently, it was found that concentrations as low as 0.004% and 0.0078% polymer antimicrobial respectively, were able to inhibit *C. albicans* both in dispersed, planktonic and biofilm form after 24, 48 and 72-hours (see **Table II-D**). The MBC value for *S. aureus* treated with polymer antimicrobial was higher in 48-hour biofilms, however this value dropped in 72-hour biofilms. Interestingly, MBEC values for *S. aureus* treated with polymer antimicrobial were lower in 48- and 72hour biofilms, when compared to 24-hour biofilms. *P. aeruginosa* biofilms treated with polymer antimicrobial resulted in higher MBC and MBEC values than *S. aureus* and *C. albicans*, with MBC values remaining consistent (0.0156%) in 24-, 48- and 72-hour biofilms. MBEC values for polymer antimicrobial-treated *P. aeruginosa* were higher than MBC values, however these values reduced with the maturity of the biofilm (0.125%, 0.0625% and 0.156%, respectively).

Treatment of *C. albicans* with the pre-blended antimicrobial resulted in low MBC and MBEC values, however 48- and 72-hour biofilms required higher concentrations to fully eradicate the biofilm. MBC values for the pre-blended antimicrobial-treated *S. aureus* increased with the maturity of the biofilm (0.002%, 0.00375% and 0.0075%, respectively), however MBEC values remained consistent in the 24-, 48- and 72-hour biofilms (0.002%). The pre-blended antimicrobial-treated *P. aeruginosa* resulted in higher MBC and MBEC values when compared with *S. aureus* and *C. albicans*, with 48-hour biofilms requiring a higher MBC and MBEC than 24- and 72-hour biofilms (see **Table II-D**).

	Test Substance (% w/v)											
	Pre-blended antimicrobial						Polymer antimicrobial					
	24		48		72		24		48		72	
Microorganism	MBC	MBEC	MBC	MBEC	MBC	MBEC	MBC	MBEC	MBC	MBEC	MBC	MBEC
P. aeruginosa ATCC 9027	0.015	0.031	0.0075	0.063	0.00375	0.031	0.0156	0.125	0.0156	0.0625	0.0156	0.0156
S. aureus ATCC 6538	0.002	0.002	0.00375	0.002	0.0075	0.002	0.0078	0.03125	0.03125	0.0078	0.0078	0.0078
C. albicans ATCC 10231	0.002	0.002	0.002	0.00375	0.002	0.00375	0.004	0.004	0.004	0.004	0.004	0.0078

Table II-D Minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) of the pre-blended antimicrobial and polymer antimicrobial on 24- 48- and 72-hour biofilms.

The experiment was repeated three times (n=3).

Discussion

This chapter highlights the potential effectiveness of a novel natural fibre wound dressing to aid the absorption of excessively produced proteases in chronic wounds. However, although there is a plethora of advanced wound dressing technologies available, the management of chronic wounds still remains a major health concern and economic burden in healthcare settings worldwide. One reason for this is the complex array of factors affecting the status of a wound, not only involving increased proteolytic activity, but ischemia, age, mechanical stress and infection. It is inappropriate to adopt a 'one-fits-all' approach in wound management as all chronic wounds, whether they are venous leg ulcers, pressure ulcers or diabetic foot ulcers, are indeed, complex and differ greatly from each other. Therefore, although this novel natural fibre dressing showed the sequestration of MMPs, and may help control protease-rich exudate within chronic wounds, other wound care strategies may also need to be adopted.

This study also showed the effectiveness of the polymer antimicrobial in the eradication of biofilms. The polymer antimicrobial used in this study became a popular area of research in the treatment of infection, because of its increasing use as an antiseptic worldwide, and thus, there is a plethora of *in vitro* and *in vivo* studies demonstrating its efficacy in the control of wound bioburden. With a chemical structure similar to that of chlorhexidine, another commonly used antiseptic in oral cavity care and skin disinfection, the polymer antimicrobial used in this study has been effectively used in the decontamination of wounds and decolonisation of skin. As this study used a commercially-available pre-blended antimicrobial, it was an aim to determine whether synergy occurred between the polymer antimicrobial and other potential biofilm-disrupting technologies such as a chealting agent and surfactants in the eradication of *S. aureus*, *P. aeruginosa* and *C. albicans*. Chelating agents have indeed been linked to bacterial biofilm formation with *in vitro* evidence to suggest that sub-inhibitory doses of EGTA can either augment or prevent *S. aureus* biofilm formation depending on the strain (Abraham et al. 2012). Although the work presented in this chapter does not culture biofilms in an EGTA-rich environment, and instead a chelating agent is used to treat the biofilm, the chelating agent had little

effect on biofilm eradication. Furthermore, the potential use of chelating agent-incorporated wound dressings for infected wounds may encourage biofilm development rather than disrupt the biofilm. There is little literature on the effects of surfactants on biofilms. One study, in the context of biofouling and marine bacterial cultures, showed that the treatment of a polystyrene surface with a surfactant resulted in a decreased attachment and colonisation of marine cultures (Blainey and Marshall 1991). Although in this chapter, the surfactants had no effect on already established biofilms, it may prevent the colonisation of wound dressings used in infected chronic wounds, which may result in less surfaces in which the bacteria can attach and proliferate, preventing dissemination.

Limitations

The main limitation of this study concerns the focus of the sequestration of just one MMP. There are other well-documented MMPs in the context of chronic wounds and therefore the assessment of the efficacy of these dressings on those MMPs would be beneficial. Other studies into the efficacy of antimicrobial substances on biofilms *in vitro*, use 24-hour (or less) cultured biofilms, which may not reflect the maturity of biofilms that reside within the tissues of an open wound. To address this issue, the experiments in this chapter investigated potential biofilm disruptors on 24-, 48- and 72-hour biofilms. However, it is likely that the use of microtitre plates with peg lids may not be a sufficient model to investigate 72-hour biofilms. Crystal violet staining of biofilms in this chapter shows little difference in biofilm density between 48- and 72-hours and it may therefore mean that increased bacterial cell numbers and nutrient depletion, lead to bacterial cell death and reduced biofilm development.

Key points

The assessment of MMP-2 sequestration in a variety of commercially available wound dressings and a novel natural fibre dressing, resulted in the significant

sequestration of MMP-2 by the collagen-substrate dressing. There were no significant differences in the efficacy of MMP-2 sequestration between the novel natural fibre dressing and the collagen-substrate dressing, which indicates that this dressing may help reduce elevated MMP activity within chronic wounds.

Some of the potential biofilm-disruptor technologies examined in this study, including the chelating agent and surfactants A and B, failed to show biocidal activity against *P. aeruginosa*, *S. aureus* and *C. albicans* in planktonic and biofilm form. However the polymer antimicrobial showed low MBC and MBEC values along with the commercially available pre-blended antimicrobial. Furthermore, no synergy between the polymer antimicrobial and the chelating agent, surfactant A or surfactant B on the eradication of 24-hour biofilms was determined. Higher concentrations of the pre-blended antimicrobial and polymer antimicrobial alone were needed to eradicate 48- and 72-hour *P. aeruginosa* biofilms, however these concentrations were still considered low level.